PURIFIED AND ISOLATED PLATELET CALCIUM CHANNEL NUCLEIC ACIDS AND POLYPEPTIDES AND THERAPEUTIC AND SCREENING METHODS USING SAME

AN APPLICATION FOR

UNITED STATES LETTERS PATENT

By

Nadia Malouf
Durham, North Carolina

Timothy C. Nichols
Chapel Hill, North Carolina

"Express Mail" mailing number.: EV023030473US
Date of Deposit 12-20-01
I hereby certify that this paper and all papers and fees referred to herein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner of Patents, Washington, D.C
Amy J. Martin

Description

PURIFIED AND ISOLATED PLATELET CALCIUM CHANNEL NUCLEIC ACIDS AND POLYPEPTIDES AND THERAPEUTIC AND SCREENING METHODS USING SAME

Cross Reference to Related Applications

This application is based on and claims priority to United States Provisional Patent Application Serial No. 60/258,169 filed December 22, 2000, the entire contents of which are herein incorporated by reference.

Grant Statement

This work was supported by NIH grants I-P20-DE123474 and I-P60-DE 13079. Thus, the U.S. Government has certain rights in the invention.

Field of the Invention

The present invention relates generally to isolated and purified proteins and nucleic acids. More particularly, the present invention relates to isolated and purified platelet calcium channel polypeptides and isolated and purified nucleic acids encoding the same.

Table of Abbreviations

ASO	allele-specific oligonucleotide
A-T	ataxia-telangiectasia
BSA	bovine serum albumin
CDR	complementarity determining region
ECL	electrochemiluminescence
EST	expressed sequence tag
fl	full length
HAT	cell culture media comprising hypoxanthine, aminopterin, and thymidine

IMAGE	integrated molecular analysis of genomes and their expression
kDa	kilodalton(s)
KLH	keyhole limpet hemocyanin
L	liter(s)
LAT	ligation activated translation
LCR	ligase chain reaction
MAb	monoclonal antibody
MAb 1A	Monoclonal Antibody 1A
mL	milliliter(s)
NASDA™	nucleic acid sequence-based amplification
nm	nanometer(s)
nt	nucleotide(s)
ORF	open reading frame
PBS/BSA	phosphate buffered saline/bovine serum albumin
PCR	polymerase chain reaction
RACE	rapid amplification of conserved ends
r. t.	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SSCP	single strand conformation polymorphism
SDA	strand displacement activation
SNP	single nucleotide polymorphism
wt	wild type
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
VDCC	voltage dependent calcium channel
µg	microgram(s)

Background of the Invention

Calcium influx into the platelet controls important processes during platelet activation. Sage, S. O. *Exp Physiol.* (1997) 82:807-823; Mills, D. C. *Thromb Haemost.* (1996) 76:835-856. The pathway for this calcium entry is not

well understood. Recently, gating of an ADP receptor (P_{2x}) has been suggested to be responsible for rapid calcium (Ca^{2+}) influx into the platelet. Mackenzie, A. B., et al., *J Biol Chem.* (1996) 271:2879-2881; Sun B., et al., *J Biol Chem.* (1998) 273:11544-11547. Indeed, several ions, including Ca^{2+}, Mg^{2+} and Na^{+} enter when this receptor is activated. However, calcium entry via this receptor is believed not to be sufficient for the processes that take place during platelet activation. Jin, J. and Kanapuli S., *Proc Natl Acad Sci U S A.* (1998) 95:8070-8074; Kanapuli, S., *Trends Pharmacol Sci.* (1998) 19:391-394.

P_{2x} is an ionotropic channel, and its gating in other cell types, such as skeletal muscle cells, has an excitatory depolarizing effect that activates voltage dependent calcium channels (VDCCs) (Bean, B. P., *Trends Phys Sci.* (1993) 13:87-90; Surprenant A., *Trends Neurosci.* (1995) 18:224-229; Abbrachio, M. P., et al., *Pharmacol Ther.* (1994) 64:445-475. It is the activation of these calcium selective VDCCs that triggers calcium dependent events inside these cells, such as secretion and contraction. Ashcroft, F. M., *Ion channels and disease.* San Diego, California: Academic Press; (2000); Boyd, A. E., *J Cell Biochem.* (1992) 48:234-261; Hille B., *Ionic channels of excitable membranes.* Sunderland, MA: Sinauer Associate, Inc. publishers; (1992); Armstrong, C. M. and Hille, B., *Neuron* (1998) 20:371-380; Berridge, M. J., *J Physiol (Lond).* (1997) 499.2:291-306.

The presence of VDCCs in platelets is controversial (Sage, S. O., *Exp Physiol.* (1997) 82:807-823), and heretofor their expression has not been investigated at the molecular level. Thus, in view of the role of calcium influx in controlling processes during platelet activation, the identification of a VDCC polypeptide in platelets represents a long-felt and continuing need in the art.

Summary of the Invention

The present invention discloses an isolated and purified polynucleotide encoding a platelet voltage dependent calcium channel (VDCC) α_1 subunit polypeptide, an isolated and purified platelet VDCC α_1 subunit polypeptide, and a characterization of the role played by a platelet VDCC α_1 subunit polypeptide in modulating calcium transport during platelet activation. Optionally, a

polypeptide of the invention is a recombinant polypeptide. The platelet VDCC α_1 subunit polypeptide can comprise a platelet VDCC α_1S subunit polypeptide or a platelet VDCC α_1D subunit polypeptide. Preferably, a polypeptide of the present invention comprises a nucleotide or amino acid sequence selected from the sequences of any of SEQ ID NOs:1-8, 28, and 29.

The present invention also provides an isolated and purified polynucleotide that encodes a platelet VDCC α_1 subunit polypeptide that modulates the levels of calcium in platelets as well as biological activities affected thereby. Optionally, a polynucleotide of the present invention comprises a DNA molecule from a mammal, including a pig and a human. Preferably, a polynucleotide of the present invention encodes a polypeptide comprising an amino acid sequence of SEQ ID NO:2 or 4. Most preferably, an isolated and purified polynucleotide of the invention comprises a nucleotide sequence of any of SEQ ID NOs:1, 3, 5-8, 28, and 29. The present invention further provides recombinant nucleic acid molecules comprising disclosed seqeunces, including vectors and chimeric genes. Also provided are host cells comprising disclosed VDCC α_1 subunit sequences. A preferred host cell is a platelet or a megakaryoctye.

In another embodiment, the present invention provides an antibody that specifically binds a platelet VDCC α_1 subunit polypeptide as described above. SEQ ID NOs:1-8, 28, and 29 set forth nucleotide and amino acid sequences from exemplary mammals, pig and human. More preferably, the antibody of the invention specifically binds a platelet calcium channel polypeptide comprising a human or porcine platelet VDCC α_1 subunit polypeptide. Even more preferably, an antibody of the invention specifically binds a polypeptide comprising an amino acid sequence of SEQ ID NO:2 or 4. Also provided by the present invention are antibodies that specifically bind homologues or biologically equivalent platelet VDCC α_1 subunit polypeptides. Optionally, an antibody of the invention is a monoclonal antibody.

In another aspect, the present invention provides a process of producing an antibody that specifically binds a platelet VDCC α_1 subunit polypeptide as described above, the process comprising: (a) transfecting a recombinant host

cell with a polynucleotide that encodes a biologically active platelet VDCC α_1 subunit polypeptide; (b) culturing the host cell under conditions sufficient for expression of the polypeptide; (c) recovering the polypeptide; and (d) preparing the antibody to the polypeptide. SEQ ID NOs:1-8, 28, and 29 set forth nucleotide and amino acid sequences from representative mammals, human and pig. Preferably, the host cell is transfected with a polynucleotide of any of SEQ ID NOs:1, 3, 5-8, 28, and 29. Even more preferably, the present invention provides an antibody prepared according to the process described above. Also provided by the present invention is the use of homologues or biologically equivalent platelet VDCC α_1 subunit polynucleotides and polypeptides found in other mammals to produce antibodies.

Alternatively, the present invention provides a process of detecting a platelet VDCC α_1 subunit polypeptide as described above, wherein the process comprises immunoreacting the polypeptide with an antibody prepared according to the process described above, forming an antibody-polypeptide conjugate wherein the antibody specifically binds the VDCC α_1 subunit polypeptide, and detecting the conjugate.

In another aspect, the present invention provides an assay or assay kit for detecting the presence of a platelet VDCC α_1 subunit polypeptide in a biological sample, where the kit comprises a first antibody capable of immunoreacting with a platelet VDCC α_1 subunit polypeptide. Preferably, the first antibody is present in an amount sufficient to perform at least one assay. Also preferably, an assay kit of the invention further comprises a second antibody that immunoreacts with the first antibody. More preferably, the antibodies used in an assay kit of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably still, the first and second antibodies comprise an indicator, and, preferably, the indicator is a radioactive label, a fluorescent label or an enzyme.

In another embodiment, the present invention provides an assay or assay kit for detecting the presence, in a biological sample, of an antibody that specifically binds a platelet VDCC α_1 subunit polypeptide, the kit comprising a

first container containing a platelet VDCC α_1 subunit polypeptide that specifically binds the antibody, with the polypeptide present in an amount sufficient to perform at least one assay.

The present invention also provides a method for detecting a nucleic acid molecule that encodes a platelet VDCC α_1 subunit polypeptide in a biological sample. According to the method, a biological sample containing nucleic acid material is obtained, and a nucleic acid molecule of the present invention is hybridized under stringent conditions to the nucleic acid material of the sample. Under these conditions a hybridization duplex comprising a VDCC α_1 subunit sequence of the present invention and a VDCC α_1 subunit sequence of the present invention is formed. Detection of the hybridization duplex identifies a platelet VDCC α_1 subunit sequence in the biological sample.

A method is further provided for identifying a mutation conferring altered platelet VDCC α_1 subunit activity. In one embodiment, the method includes the steps of amplifying nucleic acid molecules in a sample, and then evaluating whether a mutation is present in the amplified nucleic acid molecule. In another embodiment, a platelet VDCC α_1 subunit mutation is detected by evaluating a platelet VDCC α_1 subunit subunit polypeptide in a biological sample.

Also provided is a method for detecting a polymorphism in a nucleic acid molecule that encodes a platelet VDCC α_1 subunit polypeptide. According to the method, a nucleic acid molecule in a sample is amplified using primers the selectively recognize a nucleic acid molecule encoding a platelet VDCC α_1 subunit polypeptide. A polymorphism can be identified in such an amplified platelet VDCC α_1 subunit sequence. The present invention also provides a kit for detecting a platelet VDCC α_1 subunit polymorphism.

In one embodiment, the present invention provides genetic assays based on the sequence of the platelet VDCC α_1 subunit genes. Platelet VDCC α_1 subunit sequences can be employed in the design of oligonucleotide primers suitable for the analysis of human genomic DNA. Thus, primers are used to screen for genetic variants by a number of PCR-based techniques, including single-strand conformation polymorphism (SSCP) analysis, SSCP/heteroduplex

analysis, enzyme mismatch cleavage, and direct sequence analysis of amplified exons. Similar techniques can be applied to putative 5'-regulatory regions, e.g. the putative promoters 5' of an exon of platelet VDCC α_1 subunit polypeptide. Automated methods can also be applied to the large-scale characterization of single nucleotide polymorphisms within and near a platelet VDCC α_1 subunit polypeptide.

Once genetic variants have been detected in specific populations, the present invention provides assays to detect the mutation by methods such as allele-specific hybridization, or restriction analysis of amplified genomic DNA containing the specific mutation. Again, these detection methods can be automated. In the case of genetic disease or human phenotypes caused by repeat expansion, the present invention provides an assay based on PCR of genomic DNA with oligonucleotide primers flanking the involved repeat.

In another aspect, the present invention provides methods for identifying candidate substances for an ability to modulate platelet VDCC α_1 subunit activity. According to the method, a test sample is established comprising a nucleic acid molecule encoding a platelet VDCC α_1 subunit polypeptide, or functional portion thereof. A candidate substance is administered to the test sample, and an interaction, effect, or combination thereof, of the candidate substance on the test sample is assayed. Preferably, the candidate substance is a candidate protein, a peptide, an antibody, a chemical compound, or a nucleic acid. In one embodiment, the test sample used according to the method comprises a cell culture expressing a VDCC α_1 subunit polypeptide. The present invention also provides a recombinant cell line suitable for use in such a method. In another embodiment, the screening method comprises a modulatable transcriptional regulatory sequence of a platelet VDCC α_1 subunit polypeptide-encoding sequence. In this case, a candidate substance as a modulator of platelet VDCC α_1 subunit activity is based on the amount of signal produced in relation to a control sample. An exemplary reporter gene encodes a platelet VDCC α_1 subunit polypeptide.

In still a further embodiment, this invention pertains to therapeutic methods based upon the modulation of the biological activity of platelet calcium

channel via the polynucleotides and polypeptides described herein. According to the method, an effective amount of a substance that modulates platelet VDCC α_1 subunit activity is administered to a cell whereby the acitivity of platelet VDCC α_1 subunit is modulated in a predictable manner. Preferably, administration of the therapeutic composition modulates calcium transport in a cell. In one embodiment, therapeutic methods are provided wherein a modulator of VDCC α_1 subunit activity that was identified according to the disclosed methods, such a modulator including but not limited to a protein, a peptide, an antibody, a chemical compound, and a nucleic acid. In another embodiment, gene therapy approaches are provided using an isolated and purified polynucleotide of the present invention. Such methods of modulating the biological activity of a platelet calcium channel polypeptide are also applicable in the laboratory and/or clinical setting to enhance the capability to store or otherwise manipulate platelets for therapeutic or diagnostic purposes.

In yet another aspect, the present invention provides a genetically modified animal. In one embodiment of the present invention, the genetically modified animal can comprise a pig with targeted modification of a pig platelet VDCC α_1 subunit gene and can further comprise pig strains with complete or partial functional inactivation of the platelet VDCC α_1 subunit polypeptide genes in megakaryocytes and platelets. In an alternative embodiment, a genetically modified animal in accordance with the present invention is prepared using an anti-sense or ribozyme platelet VDCC α_1 subunit construct, driven by a universal, tissue-specific, or inducible promoter, to reduce levels of individual VDCCs in megakaryocytes and platelets, thus achieving a "knock-down" of individual isoforms.

The present invention also provides animal strains with specific "knocked-in" modifications in a platelet VDCC α_1 subunit gene. This includes pigs and mice with genetically and/or functionally relevant point mutations in the VDCC genes, in addition to manipulations such as the insertion of disease-specific repeat expansions. The present invention also provides the generation of animal strains with conditional inactivation of individual or multiple platelet VDCC α_1 subunit polypeptide genes by creation of a conditional mutation.

Thus, a key aspect of this invention pertains to the discovery of novel platelet VDCC α_1 subunit polypeptides and nucleic acids. Preferred nucleotide and amino acid sequences are described in SEQ ID NOs:1-8, 28, and 29. It is thus another aspect of this invention to provide a purified and isolated platelet VDCC α_1 subunit polypeptide having a role in the biological activity of calcium transport modulation.

The foregoing aspects and embodiments have broad utility given the biological significance of calcium transport in platelets. By way of example, the foregoing aspects and embodiments are useful in the preparation of screening assays and assay kits that are used to identify compounds that affect or modulate platelet calcium channel biological activity, or that are used to detect the presence of the proteins and nucleic acids of this invention in biological samples.

Accordingly, it is an object of the present invention to provide a novel platelet VDCC α_1 subunit polypeptide, and to provide a novel polynucleotide encoding the same. The object is achieved in whole or in part by the present invention.

An object of the invention having been stated herein above, other objects will become evident as the description proceeds when taken in connection with the accompanying Figures and Laboratory Examples as best described herein below.

Brief Description of the Drawings

Figure 1A is a schematic presentation of platelet VDCC α_1 subunit polypeptides cloned from human megakaryocytes and porcine platelets. A human platelet VDCC α_1S subunit clone (hα_1S, 3130 bp) from human megakaryocytes encodes a polypeptide between III S_4 and the CO_2^- end of the human α_1S. A 400 bp platelet VDCC α_1 subunit clone encodes the intra-cytoplasmic loop between motifs II-III. A human platelet VDCC α_1D subunit (hα_1D, 2682 bp) clone from human megakaryocytes encodes a polypeptide between IIIP and CO_2^- end of the human α_1D subunit. A porcine platelet VDCC α_1S subunit polypeptide (pα_1S, 1031 bp) clone from porcine platelets encodes

a polypeptide region between IV S_3 and aa 1531 of α_1S. A porcine platelet VDCC α_1D subunit polypeptide (pα_1D, 210 bp) clone from porcine platelets encodes a polypeptide between II S_3 and II S5. ● = glutamate residue in cloned regions; + = positively charged residue in cloned regions; P = pore in cloned regions; Δ = residues that contribute to DHP binding.

Figure 1B is a schematic depicting sequences absent from human α_1S and α_1D and porcine α_1S in cloned regions (shaded areas).

Figure 1C is a photograph of gel electrophoresis of a PCR product (1033bp) from human megakaryocytes that was producing using oligonucleotides 1 and 2 (Table 3) as primers to amplify cDNA regions. The cDNA clones obtained in this reaction had two different sequences. Clones 1 and 2 (lanes 1 and 2) were α_1S, clone 3 (lane 3) was α_1D. Left lane, DNA low molecular weight markers VI (2.1 – 0.5 kb, Boehringer Mannheim, Indianapolis, Indiana)

Figure 1D is an autoradiograph of a Northern blot of mRNA from human megakaryocytes. 10 µg of mRNA/lane were probed with riboprobes synthesized from the PCR products described for Figure 1C. cmRNA synthesized from clone 1 (α_1S) was used to probe the mRNA on the left lane and cmRNA from clone 3 (α_1D) was synthesized and used to probe the mRNA on the right lane. Arrows point to a 6.5 kb transcript in α_1S and a 10.8 kb transcript in α_1D. In addition, a 2.5 kb band hybridized with both probes, which are homologous but not identical. cmRNA is antisense mRNA. Bars on right are 9.4 kb, 7.46 kb, 4.4 kb, 2.37 kb molecular weight standards.

Figure 2A is an autoradiograph of a Western blot on cultured megakaryocytes, which depicts that α_1S and α_1D subunits are expressed at the protein level in platelets and megakaryocytes. Lane a: the antiserum generated in rabbit against the peptide NEELRAIIKKIWKRTSMKLL (SEQ ID NO:27) located in the putative carboxyl end of α_1 subunits (arrow in fig. 1)] recognized its determinant in a 170 kDa polypeptide. Lane b: a monoclonal antibody, Mab 1A, previously described to recognize its determinant in α_1S from skeletal muscle was used to confirm that the 170 kDa polypeptide is α_1S. Lane c: normal rabbit IgG. Lane d: β cells from pancreas confirmed that the

anti-peptide antibody recognized its determinant in α_1D (208 kDa) in β cells. Lane e: the monoclonal antibody Mab 1A did not recognize a determinant in α_1D. Lane f: normal non-immune rabbit serum. Bars on right are 218 kDa and 125 kDa molecular weight standards.

Figure 2B is an autoradiograph of an immunoblot of immunoprecipitations on porcine platelets. The antipeptide antibody precipitated determinants in polypeptides with electrophoretic mobilities of 208 kDa and 170kDa (lane a, arrows). The primary antibody was replaced with non-immune rabbit IgG in the immunoprecipitation control condition (lane b). Bars on right are 218 kDa and 125 kDa molecular weight standards.

Figure 3 depicts a nucleotide sequence alignment of a human platelet VDCC α_1S subunit gene fragment of SEQ ID NO:5 and three closely related sequences, GenBank Accession No. XM_001910 (SEQ ID NO:9), GenBank Accession No. NM_00069 (SEQ ID:11), and GenBank Accession No. L33798 (SEQ ID NO:13).

Figure 4 depicts a nucleotide sequence alignment of a porcine platelet VDCC α_1S subunit gene fragment of SEQ ID NO:7 and a human VDCC α_1S subunit fragment sequence, GenBank Accession No. XM_001910 (SEQ ID NO:9).

Figure 5 depicts a nucleotide sequence alignment of a human platelet VDCC α_1D subunit fragment of SEQ ID NO:6 and three closely related sequences, GenBank Accession No. XM_003238 (SEQ ID NO:15), GenBank Accession No. NM_000720 (SEQ ID:17), and GenBank Accession No. M83566 (SEQ ID NO:19).

Detailed Description of the Invention

Disclosed herein is the first evidence for the expression of VDCC α_1 subunits in platelets. The present invention teaches novel members of the VDCC gene family that are expressed in platelets. Representative embodiments are set forth in SEQ ID NOs:1-8, 28, and 29.

Thus, the present invention pertains to isolated and purified nucleic acids encoding platelet VDCC α_1 subunit polypeptides, to isolated and purified platelet VDCC α_1 subunit polypeptides, to the characterization of the role

played by the platelet VDCC α_1 subunit polypeptides in modulating calcium levels within and outside cells, and to the characterization of upstream or downstream processes affected by such modulation (e.g. inside-out and/or outside-in signaling).

Summarily, the identification of sequences that encode platelet VDCC α_1 subunit polypeptides, the cloning of the corresponding cDNAs, and the expression of the corresponding proteins affords the molecular tools required for modulating calcium homeostasis in platelets, and has application in the development of diagnostic, pharmacological and/or therapeutic applications, including treatments for various bleeding, thrombotic, and related disorders in human and animal subjects.

Following long-standing patent law convention, the terms "a" and "an" mean "one or more" when used in this application, including the claims.

A. General Considerations

Diversity among VDCCs, expressed in their biophysical and pharmacological properties, has led to their classification into several categories (Birnbaumer L., et al., *Neuron* (1994) 13:505-506). The long-activating (L-type) dihydropyridine (DHP) sensitive VDCC is such a channel. cDNA cloning of its major, pore forming α_1 subunit from skeletal muscle, α_1S, has led to a proposed structural model for all α_1 subunits (Tanabe T., et al., *Nature* (1987) 328:313-318 and Fig. 1). This model predicts that the encoded polypeptide contains four homologous but not identical tandem motifs (I-IV) which are made up of six transmembrane regions (S_1 - S_6) each. This α_1 subunit contains sequences that convey to the channel calcium selectivity, voltage and pharmacological sensitivities, gating properties, and susceptibility to bind with other subunits and neighboring proteins. (Hille, B., *Ionic channels of excitable membranes*, Sunderland, MA: Sinauer Associate, Inc. Publishers (1992); Peres-Reyes, E. and Schneider, T., *Kidney Int.* (1995) 48:1111-1124). While α_1S is the major subunit from L-type VDCC in skeletal muscle, α_1D is that from neuro-endocrine cells (Birnbaumer L., et al., *Neuron* (1994) 13:505-506; Seino, S., et al., *Proc Natl Acad Sci U S A.* (1992) 89:584-588).

In accordance with the present invention, the expression and ultrastructural localization of the VDCCs in platelets and their progenitor cell,

the megakaryocyte, has been investigated. The novel polypeptides and polynucleotides disclosed herein represent the first identification of a VDCC in platelets and megakaryocytes.

The gene structure of platelet VDCC α_1 subunits derived from human megakaryocytes and porcine platelets is depicted in Figure 1. The characteristic feature of platelet VDCC α_1 subunits as compared with known VDCC α_1 subunits is indicated as a missing sequence in the IV $S_3 - S_4$ linker. Figure 2 demonstrates that platelet VDCC α_1S and α_1D subunit polypeptides are expressed in platelets and megakaryocytes.

B. Polypeptides and Polynucleotides

As used in the following detailed description and in the claims, the term "platelet VDCC" includes nucleic acids and polypeptides encoding calcium channels in platelets and megakaryocytes. Platelet VDCC α_1S subunit nucleic acid sequences are expressed in platelets and megakaryotcytes, and optionally in other tissues. The term "platelet VDCC" specifically refers to a VDCC α_1 subunit characterized by the IV $S_3 - S_4$ linker. Particularly, a platelet VDCC α_1S subunit polypeptide and a platelet VDCC α_1D subunit polypeptide are disclosed herein. Representative platelet VDCC α_1S subunits from human and porcine sources are disclosed in SEQ ID NOs:1, 2, 5, and 28 and SEQ ID NOs:7-8, respectively. Representative human platelet VDCC α_1D subunits are disclosed in SEQ ID NOs:3, 4, 6, and 29.

The term "platelet VDCC" further comprises vertebrate homologues of platelet calcium channel family members, including, but not limited to, mammalian and avian homologues. Representative mammalian homologues of platelet calcium channel family members include, but are not limited to, porcine and human homologues. VDCC α_1 subunit homologues are characterized by missing sequences that encode the IV $S_3 - S_4$ linker region of VDCC α_1S polypeptides, as disclosed herein for human VDCC α_1S subunit, porcine VDCC α_1S subunit, and human VDCC α_1D subunit.

The terms "platelet VDCC α_1 subunit gene product", "platelet VDCC α_1 subunit protein", and "platelet VDCC α_1 subunit polypeptide" refer to peptides having amino acid sequences which are substantially identical to native amino acid sequences from the organism of interest and which are biologically active

in that they comprise the amino acid sequence of a platelet VDCC α_1 subunit polypeptide, or cross-react with antibodies that specifically bind a platelet VDCC α_1 subunit polypeptide, or retain all or some of the biological activity of the native amino acid sequence or protein. Such biological activity can include immunogenicity. As disclosed herein below, an important feature of platelet VDCC α_1 subunits is the absence of sequences encoding a portion of the IV S_3 – S_4 linker of VDCC α_1 subunit polypeptides.

The terms "platelet VDCC α_1 subunit gene product", "platelet VDCC α_1 subunit protein", and "platelet VDCC α_1 subunit polypeptide" are preferably meant to encompass a platelet VDCC α_1S subunit polypeptide or a platelet VDCC α_1D subunit polypeptide, including but not limited to those disclosed herein as SEQ ID NOs:2 and 4. Indeed, the definitions and explanations of "platelet VDCC α_1 subunit gene product", "platelet VDCC α_1 subunit protein", and "platelet VDCC α_1 subunit polypeptide" presented herein are also meant to be applied to a platelet VDCC α_1S subunit polypeptide or a platelet VDCC α_1D subunit polypeptide.

The terms "platelet VDCC α_1 subunit gene product", "platelet VDCC α_1 subunit protein", and "platelet VDCC α_1 subunit polypeptide" also include analogs of a platelet VDCC α_1 subunit polypeptide. By "analog" is intended that a DNA or peptide sequence can contain alterations relative to the sequences disclosed herein, yet retain all or some of the biological activity of those sequences. Analogs can be derived from nucleotide sequences as are disclosed herein or from other organisms, or can be created synthetically. Those skilled in the art will appreciate that other analogs, as yet undisclosed or undiscovered, can be used to design and/or construct calcium channel analogs. There is no need for a "platelet VDCC α_1 subunit gene product", "platelet VDCC α_1 subunit protein", and "platelet VDCC α_1 subunit polypeptide" to comprise all or substantially all of the amino acid sequence of a native platelet VDCC α_1 subunit polypeptide gene product. Shorter or longer sequences are anticipated to be of use in the invention; shorter sequences are herein referred to as "segments." Thus, the terms "platelet VDCC α_1 subunit gene product", "platelet VDCC α_1 subunit protein", and "platelet VDCC α_1 subunit polypeptide" also include fusion or recombinant platelet VDCC α_1

subunit polypeptides and proteins comprising sequences of the present invention. Methods of preparing such proteins are known in the art.

The terms "platelet VDCC α_1 subunit gene ", "platelet VDCC α_1 subunit gene segment", "platelet VDCC α_1 subunit gene sequence", "platelet VDCC α_1 subunit polynucleotide", "platelet VDCC α_1 subunit nucleic acid molecule", and "platelet VDCC α_1 subunit nucleic acid sequence" refer to any nucleic acid sequence (e.g. a DNA sequence) that is substantially identical to a polynucleotide sequence encoding a platelet VDCC α_1 subunit gene product, platelet VDCC α_1 subunit protein, or platelet VDCC α_1 subunit polypeptide as defined above, and can also comprise any combination of associated control sequences. The terms also refer to RNA, or antisense sequences, complementary to such DNA sequences. As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Furthermore, a DNA segment encoding a platelet VDCC α_1 subunit polypeptide refers to a DNA segment that contains platelet VDCC α_1 subunit coding sequences, yet is isolated away from, or purified free from, total genomic DNA of a source species, such as *Homo sapiens*. Included within the term "DNA segment" are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phages, viruses, and the like.

The terms "platelet VDCC α_1 subunit gene ", "platelet VDCC α_1 subunit gene segment", "platelet VDCC α_1 subunit gene sequence", "platelet VDCC α_1 subunit polynucleotide", "platelet VDCC α_1 subunit nucleic acid molecule", and "platelet VDCC α_1 subunit nucleic acid sequence" are preferably meant to encompass a polynucleotide encoding a platelet VDCC α_1S subunit polypeptide or a platelet VDCC α_1D subunit polypeptide, including but not limited to those disclosed herein as SEQ ID NOs:1, 3, 5-8, 28, and 29. Indeed, the definitions and explanations of the terms "platelet VDCC α_1 subunit gene", "platelet VDCC α_1 subunit gene segment", "platelet VDCC α_1 subunit gene sequence", "platelet VDCC α_1 subunit polynucleotide", "platelet VDCC α_1 subunit nucleic acid molecule", and "platelet VDCC α_1 subunit nucleic acid sequence" presented herein are also meant to be applied to a platelet VDCC α_1S subunit polypeptide or a platelet VDCC α_1D subunit polypeptide.

A characteristic feature of the platelet VDCC α_1S subunits of the present invention is the absence of sequences that encode a portion of the IV $S_3 - S_4$ linker of known VDCC α_1S subunits. The sequences that are lacking in the disclosed platelet VDCC α_1S subunits are set forth as SEQ ID NOs:23-24 (α_1S) and SEQ ID NOs:25-26 (α_1D). The absence of these sequences from the platelet VDCC α_1S subunits of the present invention is depicted in Figures 1A, 1B, and 3-5.

Figures 1A and 1B show a schematic represention of the sequences that are absent from human and porcine platelet VDCC α_1 subunits in the region of the IV $S_3 - S_4$ linker.

Figure 3 depicts a nucleotide sequence alignment of a human platelet VDCC α_1S subunit fragment of SEQ ID NO:5 and three closely related sequences, GenBank Accession No. XM_001910 (SEQ ID NO:9), GenBank Accession No. NM_00069 (SEQ ID:11), and GenBank Accession No. L33798 (SEQ ID NO:13). The nucleotide sequence of SEQ ID NO:5 represents an individual sequence read of a cloned human platelet VDCC α_1S subunit fragment. When using this fragment as a query sequence, BLAST analysis indicated the three above-mentioned GenBank sequences as haivng the highest level of sequence identity with platelet VDCC α_1S subunit sequences of the present invention. The alignment further reveals the absence of an about 57 base pair sequence (SEQ ID NO:23) in the human platelet VDCC α_1S subunit fragment of SEQ ID NO:5 compared to known VDCC α_1S subunit sequences. The deleted sequence is revealed by disruption of each alignment between contiguous base pairs 48 and 49 of the query sequence, and substantial sequence conservation is observed over the remainder of the query sequence.

Figure 4 depicts a nucleotide sequence alignment of a porcine platelet VDCC α_1S subunit gene fragment of SEQ ID NO:7 and a human VDCC α_1S subunit fragment sequence, GenBank Accession No. XM_001910 (SEQ ID NO:9). The nucleotide sequence of SEQ ID NO:7 represents an individual sequence read of a cloned porcine platelet VDCC α_1S subunit fragment. When using this fragment as a query sequence, BLAST analysis indicated human VDCC α_1S subunit gene (GenBank Accession No. 001910, SEQ ID NO:9) and

rabbit dihydropyridine receptor (GenBank Accesssion No. X05921, SEQ ID NO:21) as being the most closely related sequences (Table 1). The alignment further reveals the absence of an about 57 base pair sequence (SEQ ID NO:23) in the porcine platelet VDCC α_1S subunit fragment of SEQ ID NO:7, as the alignment is disrupted between base pairs 60 and 67 base pairs of the query sequence, but substantial sequence conservation is observed over the remainder of the query sequence.

Table 1

SEQ ID NO.	applicants' reference	best BLAST hit (ACCESSION)	Score (bits)	E value	Identities
7	990210Db29 pa1S1511-1325	XM 001910	761	0.0	516/599 92%
8	990208Cb18 pa1S1172-1365	X05921	563	0.0	395/432 91%

A comparison of the predicted amino acid sequences encoded by the human and porcine platelet VDCC α_1S subunit fragments demonstrates that the deleted seqeunce, relative to known VDCC α_1S subunit sequences, is similarly positioned in human and porcine platelet VDCC α_1S subunit sequences (Fig. 1A and 1B).

Figure 5 depicts a nucleotide sequence alignment of a human platelet VDCC α_1D subunit gene fragment of SEQ ID NO:6 and three closely related sequences, GenBank Accession No. XM_003238 (SEQ ID NO:15), GenBank Accession No. NM_000720 (SEQ ID:17), and GenBank Accession No. M83566 (SEQ ID NO:19). The nucleotide sequence of SEQ ID NO:6 represents an individual sequence read of a cloned human platelet VDCC α_1D subunit fragment. When using this fragment as a query sequence, BLAST analysis indicated the three above-mentioned GenBank sequences as having the highest level of sequence identity with platelet VDCC α_1D subunit sequences of the present invention. The alignment further reveals the absence of an about 48 base pair sequence (SEQ ID NO:25) in the human platelet VDCC α_1D subunit fragment of SEQ ID NO:6 as compared to known sequences. The deleted sequence is revealed by disruption or each alignment between contiguous base pairs 44 and 49 of the query sequence. Substantial sequence conservation is observed over the remainder of the query sequence.

B.1. Sequence Similarity and Identity

As used herein, the term "substantially similar" means that a particular sequence varies from a platelet VDCC α_1 subunit nucleic acid sequence, or a platelet VDCC α_1 subunit amino acid sequence by one or more deletions, substitutions, or additions, the net effect of which is to retain at least some of biological activity of the natural gene, gene product, or sequence. For example, a "substantially similar" polypeptide can exhibit decreased or increased biological activity, as in a pathological or disease- or dysfunction-causing condition. Such sequences include "mutant" or "polymorphic" sequences, or sequences in which the biological activity is altered to some degree but retains at least some of the original biological activity. A critical measure of substantial identity of a platelet VDCC α_1 subunit is the absence of nucleotide sequences encoding and amino acid sequences comprising (SEQ

ID NOs:23-26) a region of the IV S_3 – S_4 linker of VDCC α_1 subunit polypeptides.

Nucleic acids that are substantially identical to SEQ ID NOs:1, 3, 5-8, 28, and 29 are preferred platelet VDCC α_1 subunit sequences, e.g. allelic variants, genetically altered versions of the gene, etc., bind to the provided platelet VDCC α_1 subunit sequences under stringent hybridization conditions. By using probes, particularly labeled probes of DNA sequences, one can isolate homologous or related genes. The source of homologous genes can be any species, e.g. primate species, rodents (such as rats and mice), canines, felines, bovines, equines, etc.

Between mammalian species, e.g. human and pig, homologues have substantial sequence similarity, i.e. at least 75% sequence identity between nucleotide sequences, and more preferably at least 90% sequence identity. Sequence similarity is calculated based on a reference sequence, which can be a subset of a larger sequence, such as a conserved motif, coding region, flanking region, etc. A reference sequence will usually be at least about 18 nt long, more usually at least about 30 nt long, and can extend to the complete sequence that is being compared. Algorithms for sequence analysis are known in the art, such as BLAST, described in Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10. In determining nucleic acid sequences, all subject nucleic acid sequences capable of encoding substantially similar amino acid sequences are considered to be substantially similar to a reference nucleic acid sequence, regardless of differences in codon sequences or substitution of equivalent amino acids to create biologically functional equivalents.

Percent identity or percent similarity of a DNA or peptide sequence can be determined, for example, by comparing sequence information using the GAP computer program, available from the University of Wisconsin Geneticist Computer Group. The GAP program utilizes the alignment method of Needleman *et al.*, (1970) *J. Mol. Biol.* 48: 443, as revised by Smith *et al.*,(1981) *Adv. Appl. Math.* 2: 482. Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred parameters for the GAP program are the default parameters,

which do not impose a penalty for end gaps. See Schwartz et al., eds., (1979), Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, pp. 357-358; Gribskov *et al.*, (1986) *Nucl. Acids. Res.* 14: 6745.

The term "similarity" is contrasted with the term "identity". Similarity is defined as above; "identity", however, means a nucleic acid or amino acid sequence having the same amino acid at the same relative position in a given family member of a gene family. Homology and similarity are generally viewed as broader terms than the term identity. Biochemically similar amino acids, for example leucine and isoleucine or glutamate/aspartate, can be present at the same position-- these are not identical per se, but are biochemically "similar." As disclosed herein, these are referred to as conservative differences or conservative substitutions. This differs from a conservative mutation at the DNA level, which changes the nucleotide sequence without making a change in the encoded amino acid, e.g. TCC to TCA, both of which encode serine.

As used herein, DNA analog sequences are "substantially identical" to specific DNA sequences disclosed herein if: (a) the DNA analog sequence is derived from coding regions of the nucleic acid sequence shown in any of SEQ ID NOs:1, 3, 5-8, 28, and 29 and lacks sequences of SEQ ID NOs:23 and 25; or (b) the DNA analog sequence is capable of hybridization to any of SEQ ID NOs:1, 3, 5-8, 28, and 29 under stringent conditions, lacks sequences of SEQ ID NOs:23 and 25, and encodes a biologically active gene product of the nucleic acid sequence shown in any of SEQ ID NOs:1, 3, 5-8, 28, and 29; or (c) the DNA sequences are degenerate as a result of alternative genetic code to the DNA analog sequences defined in (a) and/or (b). Substantially identical analog proteins will be greater than about 60% identical to the corresponding sequence of the native protein. Sequences having lesser degrees of identity but comparable biological activity are considered to be equivalents.

As used herein, "stringent conditions" means conditions of high stringency, for example 6XSSC, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, 0.1% sodium dodecyl sulfate, 100 g/mL salmon sperm DNA and 15% formamide at 68° C. For the purposes of specifying additional conditions of high stringency, preferred conditions are salt concentration of about 200 mM and temperature of about 45° C. One example of such stringent

conditions is hybridization at 4XSSC, at 65° C, followed by a washing in 0.1XSSC at 65° C for one hour. Another exemplary stringent hybridization scheme uses 50% formamide, 4XSSC at 42° C. Stringent features are understood to be able to detect a remoter address.

In contrast, nucleic acids having sequence similarity are detected by hybridization under lower stringency conditions. Thus, sequence identity can be determined by hybridization under lower stringency conditions, for example, at 50° C or higher and 0.1XSSC (9 mM NaCl/0.9 mM sodium citrate) and the sequences will remain bound when subjected to washing at 55° C in 1XSSC.

Thus, in certain embodiments, the invention concerns the use of platelet VDCC α_1 subunit genes and gene products that include within their respective sequences a sequence which is essentially that of a platelet VDCC α_1 subunit gene, or the corresponding protein. The term "a sequence essentially as that of a platelet VDCC α_1 subunit gene", means that the sequence is substantially identical to a portion of a platelet VDCC α_1 subunit gene and contain a minority of bases or amino acids (whether DNA or protein) which are not identical to those of a platelet VDCC α_1 subunit protein or a platelet VDCC α_1 subunit gene, or which are not a biologically functional equivalent. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein.

Nucleotide sequences are "substantially identical" where they have between about 70% and about 80% or more preferably, between about 81% and about 90%, or even more preferably, between about 91% and about 99%, sequence identity for nucleic acid residues which are identical to the nucleotide sequence of a platelet VDCC α_1 subunit gene. Gene structure is also a useful measure of substantially identical sequences. In this case, the omission of sequences encoding a region of the IV $S_3 - S_4$ linker of VDCC α_1 subunit polypeptides is a critical feature of the disclosed sequences.

Peptide sequences which have about 35%, or 45%, or preferably from 45-55%, or more preferably 55-65%, or most preferably 65% or greater amino acids which are identical or functionally equivalent or biologically functionally equivalent to the amino acids of a platelet VDCC α_1 subunit polypeptide will be

sequences which are "substantially similar". Peptide structure is also a useful indicator of substantially identical sequences. The present invention discloses sequences that have a shorter IV $S_3 - S_4$ linker compared to known VDCC α_1 subunit polypeptides. Thus, this is an important feature when considering substantial similarity among VDCC α_1 subunit sequences.

Platelet VDCC α_1 subunit gene products and platelet VDCC α_1 subunit-encoding nucleic acid sequences which have functionally equivalent codons are also covered by the invention. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the ACG and AGU codons for serine (Table 2). Thus, when referring to the sequence examples presented in SEQ ID NOs:1, 3, 5-8, 28, and 29 applicants contemplate substitution of functionally equivalent codons of Table 2 into the sequence examples of SEQ ID NOs:1, 3, 5-8, 28, and 29. Thus, applicants are in possession of amino acid and nucleic acids sequences which include such substitutions but which are not set forth herein in their entirety for convenience.

TABLE 2 - Functionally Equivalent Codons.

Amino Acids			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic Acid	Asp	D	GAC GAU
Glumatic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU

Serine	Ser	S	ACG AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

It will also be understood by those of skill in the art that amino acid and nucleic acid sequences can include additional residues, such as additional N– or C-terminal amino acids or 5' or 3' nucleic acid sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence retains biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences which can, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or can include various internal sequences, *i.e.*, introns, which are known to occur within genes.

The present invention also encompasses the use of nucleotide segments that are complementary to the sequences of the present invention. Nucleic acid sequences which are "complementary" are those which are base-paired according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences which are substantially complementary, as can be assessed by the same nucleotide comparison set forth above, or is defined as being capable of hybridizing to the nucleic acid segment in question under moderately stringent conditions such as those described herein. A particular example of a provided complementary nucleic acid segment is an antisense oligonucleotide. In this case, complementary is defined by both cross-hybridization and the lack of coding sequences of SEQ ID NOs:23 and 25.

Hybridization can be used to assess complementary sequences and/or to isolate complementary nucleotide sequences. Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the

hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. Stringent temperature conditions will generally include temperatures in excess of about 30°C, typically in excess of about 37°C, and preferably in excess of about 45°C. Stringent salt conditions will ordinarily be less than about 1,000 mM, typically less than about 500 mM, and preferably less than about 200 mM. However, the combination of parameters is much more important than the measure of any single parameter. (See e.g., Wetmur & Davidson, 1968). Determining appropriate hybridization conditions to identify and/or isolate sequences containing high levels of identity is well known in the art. (See e.g., Sambrook *et al.*, 1989).

Representative moderate stringency conditions comprise, for example, hybridization at 50°C and in 10XSSC (0.9 M NaCl/0.09 M sodium citrate), wherein the hybridized nucleic acid molecules remain bound when subjected to washing at 55°C in 1XSSC. Sequence identity can be further determined by hybridization under more stringent conditions, for example, at 50°C or higher and 0.1XSSC (9 mM NaCl/0.9 mM sodium citrate), as described below. Typically, under "stringent conditions" a probe will hybridize specifically to its target sequence, but to no other sequences.

For the purposes of specifying conditions of high stringency, preferred conditions are salt concentration of about 200 mM and temperature of about 45°C. One example of such stringent conditions is hybridization at 4XSSC, at 65°C, followed by a washing in 0.1XSSC at 65°C for one hour. Another representative stringent hybridization scheme uses 50% formamide, 4XSSC at 42°C. As used herein, "stringent conditions" can also mean conditions of high stringency, for example 6XSSC, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, 0.1% sodium dodecyl sulfate, 100 μg/mL salmon sperm DNA and 15% formamide at 68°C.

Nucleic acids that are substantially identical to the provided platelet VDCC α_1 subunit sequences, e.g. allelic variants, genetically altered versions of the gene, polymorphic sequences, etc., bind to the provided platelet VDCC α_1 subunit sequences under stringent hybridization conditions. By using probes, particularly labeled probes of DNA sequences, one can isolate homologous or related genes. The source of homologous genes can be any

species, e.g. primate species, rodents (such as rats and mice), canines, felines, bovines, ovines, equines, etc.

Between mammalian species, e.g. human and pig, homologues display similar gene structure, have substantial sequence similarity, i.e. at least 75% sequence identity between nucleotide sequences, more preferably greater than 90% sequence similarity, and specifically lack nucleotide sequences set forth as SEQ ID NOs:23 and 25. Sequence similarity is calculated based on a reference sequence, which can be a subset of a larger sequence, such as a conserved motif, coding region, flanking region, etc. A reference sequence will usually be at least about 18 nt long, more usually at least about 30 nt long, and can extend to the complete sequence that is being compared. Algorithms for sequence analysis are known in the art, such as BLAST, described in Altschul et al. (1990) *J Mol Biol* 215:403-10. Another commonly used alignment program is entitled CLUSTAL W and is described in *Nucleic Acids Res* (1994) Nov 11;22(22):4673-80, among other places. The sequences provided herein are useful for recognizing platelet VDCC α_1 subunit related and homologous proteins in database searches.

At a biological level, identity is just that, i.e. the same amino acid at the same relative position in a given family member of a gene family. Homology and similarity are generally viewed as broader terms. For example, biochemically similar amino acids, for example leucine and isoleucine or glutamate/aspartate, can be present at the same position - these are not identical per se, but are biochemically "similar". As disclosed herein, these are referred to as conservative differences or conservative substitutions. This differs from a conservative mutation at the DNA level, which changes the nucleotide sequence without making a change in the encoded amino acid, e.g. TCC to TCA, both of which encode serine.

The platelet VDCC α_1 subunit genes disclosed herein are thus homologous proteins, but when percentages are referred to herein, it is meant to refer to percent identity.

Probe sequences can also hybridize specifically to duplex DNA under certain conditions to form triplex or other higher order DNA complexes. The

preparation of such probes and suitable hybridization conditions are disclosed herein and are known in the art.

As used herein and in the claims, the term "gene" refers to both genomic sequences and cDNA sequences. "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a platelet VDCC α_1 subunit gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

Thus, in particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences which encode a platelet VDCC α_1 subunit polypeptide that includes within its amino acid sequence an amino acid sequence of the present invention. In other particular embodiments, the invention concerns recombinant vectors incorporating DNA segments which encode a protein comprising the amino acid sequence of a human platelet VDCC α_1 subunit polypeptide protein.

B.2. Biologically Functional Equivalents

As mentioned above, modifications and changes can be made in the structure of the platelet VDCC α_1 subunit polypeptide proteins and peptides described herein and still constitute a molecule having like or otherwise desirable characteristics. For example, certain amino acids can be substituted for other amino acids in a protein structure without appreciable loss of interactive capacity with, for example, structures in the nucleus of a cell. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or the nucleic acid sequence encoding it) to obtain a protein with the same, enhanced, or antagonistic properties. Such properties can be achieved by interaction with the normal targets of the native protein, but this need not be the case, and the biological activity of the invention is not limited to a particular mechanism of action. It is thus provided in accordance with the present invention that various changes can be made in

the sequence of the platelet VDCC α_1 subunit polypeptide proteins and peptides or underlying nucleic acid sequence without appreciable loss of their biological utility or activity.

Biologically functional equivalent peptides, as used herein, are peptides in which certain, but not most or all, of the amino acids can be substituted. Thus, when referring to the sequence examples presented in SEQ ID NOs:1, 3, 5-8, 28, and 29, applicants envision substitution of codons that encode biologically equivalent amino acids as described herein into the sequence examples of SEQ ID NOs:1, 3, 5-8, 28, and 29. Thus, applicants are in possession of amino acid and nucleic acids sequences which include such substitutions but which are not set forth herein in their entirety for convenience.

Alternatively, functionally equivalent proteins or peptides can be created via the application of recombinant DNA technology, in which changes in the protein structure can be engineered, based on considerations of the properties of the amino acids being exchanged, e.g. substitution of Ile for Leu. Changes designed by man can be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the antigenicity of the protein or to test platelet VDCC α_1 subunit mutants in order to examine platelet VDCC α_1 subunit calcium transport activity, or other activity at the molecular level.

Amino acid substitutions, such as those which might be employed in modifying the platelet VDCC α_1 subunit polypeptide proteins and peptides described herein, are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all of similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents. Other biologically functionally equivalent changes will be appreciated by those of skill in the art.

In making biologically functional equivalent amino acid substitutions, the hydropathic index of amino acids can be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+ 4.5); valine (+ 4.2); leucine (+ 3.8); phenylalanine (+ 2.8); cysteine (+ 2.5); methionine (+ 1.9); alanine (+ 1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 of the original value is preferred, those which are within ±1 of the original value are particularly preferred, and those within ±0.5 of the original value are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.* with a biological property of the protein. It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent protein.

As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+ 3.0); lysine (+ 3.0); aspartate (+ 3.0±1); glutamate (+ 3.0±1); serine (+ 0.3); asparagine (+ 0.2); glutamine (+ 0.2); glycine (0); threonine (-0.4); proline (-0.5±1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4) .

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 of the

original value is preferred, those which are within ±1 of the original value are particularly preferred, and those within ±0.5 of the original value are even more particularly preferred.

While discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes can be effected by alteration of the encoding DNA, taking into consideration also that the genetic code is degenerate and that two or more codons can code for the same amino acid.

Thus, it will also be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NOs:1-8, 28, and 29. Recombinant vectors and isolated DNA segments can therefore variously include the platelet VDCC α_1 subunit polypeptide-encoding region itself, coding regions bearing selected alterations or modifications in the basic coding region, or larger polypeptides which nevertheless comprise platelet VDCC α_1 subunit polypeptide-encoding regions or can encode biologically functional equivalent proteins or peptides. Biological activity of a platelet VDCC α_1 subunit polypeptide can be determined, for example, by any of the assays disclosed herein below in Section G.2.

In particular embodiments, the invention concerns gene therapy methods that use isolated DNA segments and recombinant vectors incorporating DNA sequences which encode a protein comprising an amino acid sequence of SEQ ID NO:2 or 4 In other particular embodiments, the invention concerns isolated DNA sequences and recombinant DNA vectors incorporating DNA sequences which encode a protein comprising an amino acid sequence of a platelet VDCC α_1 subunit polypeptide protein from human or pig. In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that comprise a nucleic acid sequence essentially as set forth in any of SEQ ID NOs:1, 3, 5-8, 28, and 29.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, can be combined with other DNA sequences, such as promoters, enhancers, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length can vary considerably. Thus, a nucleic acid

fragment of almost any length can be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments can be prepared which include a short stretch complementary to a nucleic acid sequence set forth in any of SEQ ID NOs:1, 3, 5-8, 28, and 29, such as about 10 nucleotides, and which are up to 10,000 or 5,000 base pairs in length, with segments of 3,000 being preferred in certain cases. DNA segments with total lengths of about 4,000, 3,000, 2,000, 1,000, 500, 200, 100, and about 50 base pairs in length are also useful.

The DNA segments of the present invention encompass biologically functional equivalent platelet VDCC α_1 subunit polypeptides. Such sequences can rise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides can be created via the application of recombinant DNA technology, in which changes in the protein structure can be engineered, based on considerations of the properties of the amino acids being exchanged. Changes can be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein or to test platelet VDCC α_1 subunit mutants in order to examine activity in the modulation of calcium transport, or other activity at the molecular level. Site-directed mutagenesis techniques are known to those of skill in the art and are disclosed herein.

The invention further encompasses fusion proteins and peptides wherein the platelet VDCC α_1 subunit polypeptide coding region is aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes.

Recombinant vectors form important further aspects of the present invention. Particularly useful vectors are those in which the coding portion of the DNA segment is positioned under the control of a promoter. The promoter can be that naturally associated with the platelet VDCC α_1 subunit polypeptide gene, as can be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant

cloning and/or PCR technology and/or other methods known in the art, in conjunction with the compositions disclosed herein.

In other embodiments, certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is a promoter that is not normally associated with a platelet VDCC α_1 subunit polypeptide gene in its natural environment. Such promoters can include promoters isolated from bacterial, viral, eukaryotic, or mammalian cells. Representative heterologous promoters can thus comprise PF4, $\alpha_{IIb/\beta3}$ (GPIIb/IIIa), P-selectin, or GPIb promoter, which have been disclosed in the art. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology (for example, see Sambrook *et al.*, 1989, specifically incorporated herein by reference). The promoters employed can be constitutive or inducible and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter systems provided for use in high-level expression include, but are not limited to, the vaccinia virus promoter and the baculovirus promoter.

In an alternative embodiment, the present invention provides an expression vector comprising a polynucleotide that encodes a biologically active platelet VDCC α_1 subunit polypeptide in accordance with the present invention. Also preferably, an expression vector of the present invention comprises a polynucleotide that encodes human or pig platelet VDCC α_1 subunit polypeptide gene product. More preferably, an expression vector of the present invention comprises a polynucleotide that encodes a polypeptide comprising an amino acid residue sequence of SEQ ID NO:2 or 4. More preferably, an expression vector of the present invention comprises a polynucleotide comprising the nucleotide sequence of any of SEQ ID NOs:1, 3, 5-8, 28, and 29. Even more preferably, an expression vector of the invention comprises a polynucleotide operatively linked to an enhancer-promoter. More

preferably still, an expression vector of the invention comprises a polynucleotide operatively linked to a prokaryotic promoter. Alternatively, an expression vector of the present invention comprises a polynucleotide operatively linked to an enhancer-promoter that is a eukaryotic promoter and the expression vector further comprises a polyadenylation signal that is positioned 3' of the carboxy-terminal amino acid and within a transcriptional unit of the encoded polypeptide. The expression vector can further comprise an intronic sequence from a platelet VDCC α1 subunit gene, another platelet or megakaryocytic intronic sequence, or any other intronic sequence, as it has been shown that the inclusion of an intronic sequence into an expression vector can increase levels of expression.

In yet another embodiment, the present invention provides a recombinant host cell transfected, infected or adsorbed with a polynucleotide that encodes a biologically active platelet VDCC α_1 subunit polypeptide in accordance with the present invention. SEQ ID NOs:1-8, 28, and 29 set forth nucleotide and amino acid sequences from representative vertebrates, human and pig. Also provided by the present invention are homologous or biologically functionally equivalent polynucleotides and platelet VDCC α_1 subunit polypeptides found in other vertebrates, including particularly dog and bovine homologues. Preferably, a recombinant host cell of the present invention is transfected with the polynucleotide that encodes human or pig platelet VDCC α_1 subunit polypeptide. More preferably, a recombinant host cell of the present invention is transfected with the polynucleotide sequence encoding or set forth in any of SEQ ID NOs:1-8, 28, and 29. Even more preferably, a recombinant host cell is a mammalian cell. Most preferably, a recombinant host cell is a platelet or a megakaryocyte.

In another aspect, a recombinant host cell of the present invention is a prokaryotic host cell, including parasitic and bacterial cells. Preferably, a recombinant host cell of the invention is a bacterial cell, preferably a strain of *Escherichia coli*. More preferably, a recombinant host cell comprises a polynucleotide under the transcriptional control of regulatory signals functional in the recombinant host cell, wherein the regulatory signals appropriately

control expression of the platelet VDCC α_1 subunit polypeptide in a manner to enable all necessary transcriptional and post-transcriptional modification.

In yet another embodiment, the present invention provides a method of preparing a platelet VDCC α_1 subunit polypeptide comprising transfecting a cell with polynucleotide that encodes a biologically active platelet VDCC α_1 subunit polypeptide in accordance with the present invention, to produce a transformed host cell, and maintaining the transformed host cell under biological conditions sufficient for expression of the polypeptide. The polypeptide can be isolated if desired, using any suitable technique. The host cell can be a prokaryotic or eukaryotic cell. Preferably, the prokaryotic cell is a bacterial cell of *Escherichia coli*. More preferably, a polynucleotide transfected into the transformed cell comprises the nucleotide base sequence of any of SEQ ID NOs:1, 3, 5-8, 28, and 29. SEQ ID NOs:1-8, 28, and 29 set forth nucleotide and amino acid sequences for representative vertebrates, human and pig. Also provided by the present invention are homologues or biologically equivalent platelet VDCC α_1 subunit polypeptide polynucleotides and polypeptides found in other vertebrates, particularly warm blooded vertebrates, more particularly mammals, and even more particularly bovine and rat homologues.

As mentioned above, in connection with expression embodiments to prepare recombinant platelet VDCC α_1 subunit polypeptide proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire platelet VDCC α_1 subunit polypeptide protein, functional domains or cleavage products thereof, being most preferred. However, it will be appreciated that the use of shorter DNA segments to direct the expression of platelet VDCC α_1 subunit polypeptides or core regions, such as can be used to generate anti-platelet VDCC α_1 subunit polypeptide antibodies, also falls within the scope of the invention.

DNA segments which encode peptide antigens from about 15 to about 50 amino acids in length, or more preferably, from about 15 to about 30 amino acids in length are contemplated to be particularly useful. DNA segments encoding peptides will generally have a minimum coding length in the order of about 45 to about 150, or to about 90 nucleotides. DNA segments encoding full length proteins can have a minimum coding length on the order of about

4,000 or 5,000 nucleotides for a protein in accordance with any of SEQ ID NOs:1, 3, 5-8, 28, and 29. DNA segments of the present invention can contain 300, 400, 500, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500, or up to 5,000 nucleotides. Peptides of the present invention can contain 10, 20, 50, 100, 200, 300, 400, 500, 750, 1,000, or up to 1,500 amino acids.

B.3. Sequence Modification Techniques

Modifications to the platelet VDCC α_1 subunit proteins and peptides described herein can be carried out using techniques known in the art, including site directed mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants; for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 30 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications (*e.g.*, Adelman *et al.*, (1983) DNA 2:183; Sambrook et al., 1989) and can be achieved in a variety of ways generally known to those of skill in the art.

B.4. Other Structural Equivalents

The knowledge of the structure of the platelet VDCC α_1 subunit polypeptide of the present invention provides a tool for investigating the mechanism of action of these proteins in a subject. For example, binding of these proteins to various substrate molecules can be predicted by various computer models. Upon discovering that such binding in fact takes place, knowledge of the protein structure then allows design and synthesis of small

molecules which mimic the functional binding of the platelet VDCC α_1 subunit polypeptide to the substrate. This is the method of "rational" drug design, also described below.

Use of the isolated and purified platelet VDCC α_1 subunit polypeptide of the present invention in rational drug design is thus provided in accordance with the present invention. Additional rational drug design techniques are described in U.S. Patent Nos. 5,834,228 and 5,872,011, herein incorporated in their entirety.

Thus, in addition to the peptidyl compounds described herein, other sterically similar compounds can be formulated to mimic the key portions of the peptide structure. Such compounds can be used in the same manner as the peptides of the invention and hence are also functional equivalents. The generation of a structural functional equivalent can be achieved by the techniques of modeling and chemical design known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

C. Introduction of Gene Products

In accordance with the present invention, where a platelet VDCC α_1 subunit gene itself is employed to introduce a platelet VDCC α_1 subunit gene product, a convenient method of introduction will be through the use of a recombinant vector that incorporates the desired gene, together with its associated control sequences. The preparation of recombinant vectors is well known to those of skill in the art and described in many references, such as, for example, Sambrook *et al.* (1989), incorporated herein in its entirety.

C.1. Vector Construction

It is understood that the DNA coding sequences to be expressed, in this case those encoding the platelet VDCC α_1 subunit gene products, are positioned in a vector adjacent to and under the control of a promoter. It is understood in the art that to bring a coding sequence under the control of such a promoter, one generally positions the 5′ end of the transcription initiation site of the transcriptional reading frame of the gene product to be expressed between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3′ of) the chosen promoter. One can also desire to incorporate into the transcriptional

unit of the vector an appropriate polyadenylation site (*e.g.*, 5′-AATAAA-3′), if one was not contained within the original inserted DNA. Typically, these poly-A addition sites are placed about 30 to 2000 nucleotides "downstream" of the coding sequence at a position prior to transcription termination.

While use of the control sequences of the specific gene will be preferred, other control sequences can be employed, so long as they are compatible with the genotype of the cell being treated. Thus, one can mention other useful promoters by way of example, including, *e.g.*, an SV40 early promoter, a long terminal repeat promoter from retrovirus, an actin promoter, a heat shock promoter, a metallothionein promoter, and the like. Representative platelet specific promoters include but are not limited to PF4, α_{IIb} (GPIIb), P-selectin or GPIb.

As is known in the art, a promoter is a region of a DNA molecule typically within about 100 nucleotide pairs upstream of (*i.e.*, 5' to) the point at which transcription begins (*i.e.*, a transcription start site). That region typically contains several types of DNA sequence elements that are located in similar relative positions in different genes.

Another type of discrete transcription regulatory sequence element is an enhancer. An enhancer imposes specificity of time, location and expression level on a particular coding region or gene. A major function of an enhancer is to increase the level of transcription of a coding sequence in a cell that contains one or more transcription factors that bind to that enhancer. An enhancer can function when located at variable distances from transcription start sites so long as a promoter is present.

As used herein, the phrase "enhancer-promoter" means a composite unit that contains both enhancer and promoter elements. An enhancer-promoter is operatively linked to a coding sequence that encodes at least one gene product. As used herein, the phrase "operatively linked" means that an enhancer-promoter is connected to a coding sequence in such a way that the transcription of that coding sequence is controlled and regulated by that enhancer-promoter. Means for operatively linking an enhancer-promoter to a coding sequence are well known in the art; the precise orientation and location

relative to a coding sequence of interest is dependent, *inter alia,* upon the specific nature of the enhancer-promoter.

An enhancer-promoter used in a vector construct of the present invention can be any enhancer-promoter that drives expression in a cell to be transfected. By employing an enhancer-promoter with well-known properties, the level and pattern of gene product expression can be optimized. The vector can further comprise an intronic sequence from a platelet VDCC α1 subunit gene, another platelet or megakaryocytic intronic sequence, or any other intronic sequence, as it has been shown that the inclusion of an intronic sequence into an vector can increase levels of expression, particularly in the case of a gene therapy vector.

For introduction of, for example, a human platelet VDCC α_1 subunit gene, a vector construct that will deliver the gene to the affected cells is desired. Viral vectors can be used. These vectors will preferably be an adenoviral, a retroviral, a vaccinia viral vector, adeno-associated virus or Lentivirus; these vectors are preferred because they have been successfully used to deliver desired sequences to cells and tend to have a high infection efficiency. Suitable vector-platelet VDCC α_1 subunit gene constructs are adapted for administration as pharmaceutical compositions, as described herein below. Viral promoters can also be of use in vectors of the present invention, and are known in the art.

Commonly used viral promoters for expression vectors are derived from polyoma, cytomegalovirus, Adenovirus 2, and Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment that also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments can also be used, provided there is included the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

The origin of replication can be provided either by construction of the vector to include an exogenous origin, such as can be derived from SV40 or

other viral source, or can be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Where a platelet VDCC α_1 subunit gene itself is employed it will be most convenient to simply use a wild type platelet VDCC α_1 subunit gene directly. However, certain regions of a platelet VDCC α_1 subunit gene can be employed exclusively without employing an entire wild type platelet VDCC α_1 subunit gene. It is proposed that it will ultimately be preferable to employ the smallest region needed to modulate biological activity so that one is not introducing unnecessary DNA into cells which receive a platelet VDCC α_1 subunit gene construct. The biological activity of these regions can easily be determined by the assays reported herein.

C.2. Genetically Modified Cell Lines and Animals

It is also within the scope of the present invention to prepare a genetically modified cell line (e.g. platelet or megakarocyte) and/or a genetically modified animal (e.g. a transgenic non-human animal) that expresses a platelet VDCC α_1 subunit gene of the present invention, that does not express a platelet VDCC α_1 subunit gene, or that has a modified expression of a platelet VDCC α_1 subunit gene of the present invention. Preferred transgenic animals included but are not limited to mice, pigs and dogs.

Techniques for the preparation of genetically modified cell lines are disclosed herein above, and are generally known in the art. Modified megakaryocyte and platelet cell lines have utility in the study of the biological activity and in the preparation and development of laboratory and medical applications for platelets, including enhanced ability to store or otherwise manipulate platelets.

Techniques for the preparation of transgenic animals are known in the art. Exemplary techniques are described in U.S. Patent No. 5,489,742 (transgenic rats); U.S. Patent Nos. 4,736,866, 5,550,316, 5,614,396, 5,625,125 and 5,648,061 (transgenic mice); U.S. Patent No. 5,573,933 (transgenic pigs); 5,162,215 (transgenic avian species) and U.S. Patent No. 5,741,957 (transgenic bovine species), the entire contents of each of which are herein incorporated by reference.

With respect to a representative method for the preparation of a transgenic pig, cloned recombinant or synthetic DNA sequences or DNA segments encoding a platelet VDCC α_1 subunit polypeptide gene product are injected into fertilized eggs. The injected eggs are implanted in pseudo pregnant females and are grown to term to provide transgenic pigs whose cells express a platelet VDCC α_1 subunit polypeptide gene product.

Additionally, a genetically modified animal of the present invention can comprise a pig with targeted modification of the platelet VDCC α_1 subunit polypeptide gene. Pig strains with complete or partial functional inactivation of a platelet VDCC α_1 subunit polypeptide gene in megakaryocytes and/or in platelets are generated using standard techniques of site-specific recombination in embryonic stem cells. Capecchi, M. R. (1989) *Science* 244(4910):1288-92; Thomas, K. R., and Capecchi, M. R. (1990) *Nature* 346(6287):847-50; Delpire, E., et al. (1999) *Nat Genet* 22(2):192-5. Procedures analogous to those employed in the generation of a "knock-out" animal can be applied in the generation of a "knock-out" cell line.

Alternatives include the use of anti-sense or ribozyme VDCC constructs, driven by a universal or tissue-specific promoter, to reduce levels of a platelet VDCC α_1 subunit polypeptide in platelets or megakarocytes, thus achieving a "knock-down" of individual isoforms (Luyckx, V. A., et al. (1999) *Proc Natl Acad Sci U S A* 96(21):12174-9). The invention also provides the generation of animal strains with conditional or inducible inactivation of individual or multiple VDCC genes (Sauer, B. (1998) *Methods* 14(4):381-92). For example, pigs are created which lack expression of any platelet VDCC α_1 subunit polypeptide in platelets or megakaryocytes through the sequential mating of pig strains with lox-P-flanked VDCC genes with a transgenic line expressing Cre-recombinase in platelets or megakaryocytes, using a platelet specific promoter, such as PF4, α_{IIb} (GPIIb), P-selectin or GPIb (Ding, Y., et al. (1997) *J Biol Chem* 272(44):28142-8).

The present invention also provides animal strains with specific "knocked-in" modifications in a platelet VDCC α_1 subunit polypeptide gene. This includes animals with genetically (Forlino, A., et al. (1999) *J Biol Chem* 274(53):37923-31) and functionally (Kissel, H., et al. (2000) *Embo J*

19(6):1312-1326) relevant point mutations in the VDCC genes, in addition to manipulations such as the insertion of disease-specific repeat expansions (White, J. K., et al. (1997) *Nat Genet* 17(4):404-10).

D. Generation of Antibodies

In still another embodiment, the present invention provides an antibody that specifically binds a polypeptide of the present invention. Preferably, an antibody of the invention is a monoclonal antibody. Techniques for preparing and characterizing antibodies are well known in the art (See e.g., *Antibodies A Laboratory Manual*, E. Harlow and D. Lane, Cold Spring Harbor Laboratory, 1988).

The phrase "specifically (or selectively) binds to an antibody", or "specifically (or selectively) immunoreactive with", when referring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein in a heterogeneous population of proteins and other biological materials. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not show significant binding to other proteins present in the sample. Specific binding to an antibody under such conditions can require an antibody that is selected for its specificity for a particular protein. For example, antibodies raised to a protein with an amino acid sequence encoded by any of the nucleic acid sequences of the invention can be selected to obtain antibodies specifically immunoreactive with that protein and not with other proteins.

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide or polynucleotide of the present invention, and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given polypeptide or polynucleotide can vary in its immunogenicity. It is often necessary therefore to couple the immunogen (*e.g.*, a polypeptide or polynucleotide) of the present invention) with a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as

ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers.

Reagents for conjugating a polypeptide or a polynucleotide to a carrier protein are well known in the art and include glutaraldehyde, N—maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, immunogenicity to a particular immunogen can be enhanced by the use of non-specific stimulators of the immune response known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen used of the production of polyclonal antibodies varies, *inter alia*, upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen, *e.g.* subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal. The production of polyclonal antibodies is monitored by sampling blood of the immunized animal at various points following immunization. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored.

In another aspect, the present invention provides a method of producing an antibody that specifically binds a platelet VDCC α_1 subunit polypeptide, the method comprising: (a) transfecting recombinant host cells with a polynucleotide that encodes that polypeptide; (b) culturing the host cells under conditions sufficient for expression of the polypeptide; (c) recovering the polypeptide; and (d) preparing antibodies to the polypeptide. Preferably, the platelet VDCC α_1 subunit polypeptide is capable of modulating calcium levels within or outside of cells in accordance with the present invention.

A monoclonal antibody of the present invention can be readily prepared through use of well-known techniques such as the hybridoma techniques exemplified in U.S. Pat. No 4,196,265 and the phage-displayed techniques disclosed in U.S. Patent No. 5,260,203, the contents of which are herein incorporated by reference.

A typical technique involves first immunizing a suitable animal with a selected antigen (*e.g.*, a polypeptide or polynucleotide of the present invention) in a manner sufficient to provide an immune response. Rodents such as mice and rats are preferred animals. Spleen cells from the immunized animal are then fused with cells of an immortal myeloma cell. Where the immunized animal is a mouse, a preferred myeloma cell is a murine NS-1 myeloma cell.

The fused spleen/myeloma cells are cultured in a selective medium to select fused spleen/myeloma cells from the parental cells. Fused cells are separated from the mixture of non-fused parental cells, for example, by the addition of agents that block the *de novo* synthesis of nucleotides in the tissue culture media. This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants for reactivity with an antigen-polypeptides. The selected clones can then be propagated indefinitely to provide the monoclonal antibody.

By way of specific example, to produce an antibody of the present invention, mice are injected intraperitoneally with between about 1-200 μg of an antigen comprising a polypeptide of the present invention. B lymphocyte cells are stimulated to grow by injecting the antigen in association with an adjuvant such as complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*). At some time (*e.g.*, at least two weeks) after the first injection, mice are boosted by injection with a second dose of the antigen mixed with incomplete Freund's adjuvant.

A few weeks after the second injection, mice are tail bled and the sera titered by immunoprecipitation against radiolabeled antigen. Preferably, the method of boosting and titering is repeated until a suitable titer is achieved. The spleen of the mouse with the highest titer is removed and the spleen lymphocytes are obtained by homogenizing the spleen with a syringe.

Mutant lymphocyte cells known as myeloma cells are obtained from laboratory animals in which such cells have been induced to grow by a variety of well-known methods. Myeloma cells lack the salvage pathway of nucleotide biosynthesis. Because myeloma cells are tumor cells, they can be propagated

indefinitely in tissue culture, and are thus "immortal". Numerous cultured cell lines of myeloma cells from mice and rats, such as murine NS-1 myeloma cells, have been established.

Myeloma cells are combined under conditions appropriate to foster fusion with the normal antibody-producing cells from the spleen of the mouse or rat injected with the antigen/polypeptide of the present invention. Fusion conditions include, for example, the presence of polyethylene glycol. The resulting fused cells are hybridoma cells. Like myeloma cells, hybridoma cells grow indefinitely in culture.

Hybridoma cells are separated from unfused myeloma cells by culturing in a selection medium such as HAT media (hypoxanthine, aminopterin, and thymidine). Unfused myeloma cells lack the enzymes necessary to synthesize nucleotides from the salvage pathway because they are killed in the presence of aminopterin, methotrexate, or azaserine. Unfused lymphocytes also do not continue to grow in tissue culture. Thus, only cells that have successfully fused (hybridoma cells) can grow in the selection media.

Each of the surviving hybridoma cells produces a single antibody. These cells are then screened for the production of the specific antibody that specifically binds an antigen/polypeptide of the present invention. Single cell hybridomas are isolated by limiting dilutions of the hybridomas. The hybridomas are serially diluted many times and, after the dilutions are allowed to grow, the supernatant is tested for the presence of the monoclonal antibody. The clones producing that antibody are then cultured in large amounts to produce an antibody of the present invention in convenient quantity.

By use of a monoclonal antibody of the present invention, specific polypeptides and polynucleotide of the invention can be recognized as antigens, and thus identified. Once identified, those polypeptides and polynucleotide can be isolated and purified by techniques such as antibody-affinity chromatography. In antibody-affinity chromatography, a monoclonal antibody is bound to a solid substrate and exposed to a solution containing the desired antigen. The antigen is removed from the solution through an immunospecific reaction with the bound antibody. The polypeptide or polynucleotide is then easily removed from the substrate and purified.

E. Detecting a Polynucleotide or a Polypeptide of the Present Invention.

Alternatively, the present invention provides a method of detecting a polypeptide of the present invention, wherein the method comprises immunoreacting the polypeptides with antibodies prepared according to the method described above to form antibody-polypeptide conjugates, and detecting the conjugates.

In yet another embodiment, the present invention provides a method of detecting messenger RNA transcripts that encode a polypeptide of the present invention, wherein the method comprises hybridizing the messenger RNA transcripts with polynucleotide sequences that encode the polypeptide to form duplexes; and detecting the duplex. Alternatively, the present invention provides a method of detecting DNA molecules that encode a polypeptide of the present invention, wherein the method comprises hybridizing DNA molecules with a polynucleotide that encodes that polypeptide to form duplexes; and detecting the duplexes.

The detection and screening assays disclosed herein can optionally be used as a prognosis tool and/or diagnostic aid. Platelet VDCC α_1 subunit polypeptides and nucleic acids can be readily used in clinical setting as a prognostic and/ or diagnostic indicator for screening for levels of expression of platelet VDCC α_1 subunit polypeptides, or alterations in native sequences. The nucleotide sequences of the subject invention can be used to detect differences in gene or gene product sequences between normal, carrier, or affected individuals. As discussed herein above, such differences can consist of single-nucleotide changes or multiple changes, deletions, or additions in the native sequence which result in altered transcription, translation, or activity or biological activity or properties of the gene or gene product. These differences can be readily detected using the compositions of the present invention and techniques known in the art, including but not limited to SSCP analysis, RFLP analysis, or other PCR- or nucleotide-based analysis.

DNA segments of the invention or RNA having the sequence of, or a sequence complementary to, SEQ ID NOs:1, 3, 5-8, 28, and 29 can be used. Such polynucleic acids can comprise 10, 20, 40, 50, 70, 100, 250, 300, 400, 500, or 1,000 nucleotides or up to the full length of SEQ ID NOs:1, 3, 5-8, 28,

and 29. Such polynucleic acids can, but need not, encode polypeptides which retain some or all of the biological activity of the native gene or gene product.

The present invention provides a method of screening a biological sample for the presence of a platelet VDCC α_1 subunit polypeptide. A biological sample to be screened can be a biological fluid such as extracellular or intracellular fluid, or a cell or tissue extract or homogenate. A biological sample can also be an isolated cell (*e.g.*, in culture) or a collection of cells such as in a tissue sample or histology sample. A tissue sample can be suspended in a liquid medium or fixed onto a solid support such as a microscope slide.

In accordance with a screening assay method, a biological sample is exposed to an antibody that specifically binds the polypeptide whose presence is being assayed. Typically, exposure is accomplished by forming an admixture in a liquid medium that contains both the antibody and the candidate polypeptide. Either the antibody or the sample with the polypeptide can be affixed to a solid support (*e.g.*, a column or a microtiter plate). Additional details of methods for such assays are known in the art. The presence of polypeptide in the sample is detected by evaluating the formation and presence of antibody-polypeptide conjugates. Techniques for detecting such antibody-antigen conjugates or complexes are well known in the art and include but are not limited to centrifugation, affinity chromatography and the like, and binding of a secondary antibody to the antibody-candidate receptor complex.

In one embodiment, detection is accomplished by detecting an indicator affixed to the antibody. Exemplary and well-known indicators include radioactive labels (*e.g.*, ^{32}P, ^{125}I, ^{14}C), a second antibody or an enzyme such as horseradish peroxidase. Techniques for affixing indicators to antibodies are known in the art.

In another aspect, the present invention provides a method of screening a biological sample for the presence of antibodies that specifically bind a platelet VDCC α_1 subunit polypeptide. Preferably the antibody so identified has activity in the modulation of platelet VDCC α_1 subunit polypeptide biological activity in accordance with the present invention. In accordance with such a method, a biological sample is exposed to a platelet VDCC α_1 subunit polypeptide under biological conditions and for a period of time sufficient for

antibody-polypeptide conjugate formation and the formed conjugates are detected.

A DNA or RNA molecule and particularly a DNA segment or polynucleotide can be used for hybridization to a DNA or RNA source or sample suspected of encoding a platelet VDCC α_1 subunit polypeptide of the present invention; such molecules are referred to as "probes", and such hybridization is "probing". Such probes can be made synthetically. The probing is usually accomplished by hybridizing the oligonucleotide to a DNA source suspected of possessing a platelet VDCC α_1 subunit polypeptide gene. In some cases, the probes constitute only a single probe, and in others, the probes constitute a collection of probes based on a certain amino acid sequence or sequences of the polypeptide and account in their diversity for the redundancy inherent in the genetic code.

Other molecules which are neither DNA nor RNA but are capable of hybridizing in a similar manner and which are designed structurally to mimic the DNA or RNA sequence of a platelet VDCC α_1 subunit polypeptide gene are also provided. Here, a suitable source to examine is capable of expressing a polypeptide of the present invention and can be a genomic library of a cell line of interest. Alternatively, a source of DNA or RNA can include total DNA or RNA from the cell line of interest. Once the hybridization method of the invention has identified a candidate DNA segment, a positive clone can be confirmed by further hybridization, restriction enzyme mapping, sequencing and/or expression and testing.

Alternatively, such DNA molecules can be used in a number of techniques including their use as: (1) diagnostic tools to detect normal and abnormal DNA sequences in DNA derived from patient's cells; (2) reagents for detecting and isolating other members of the polypeptide family and related polypeptides from a DNA library potentially containing such sequences; (3) primers for hybridizing to related sequences for the purpose of amplifying those sequences; (4) primers for altering native platelet VDCC α_1 subunit DNA sequences; as well as (5) other techniques which rely on the similarity of the sequences of interest to those of the sequences herein disclosed.

As set forth above, in certain aspects, DNA sequence information provided by the invention allows for the preparation of probes that specifically hybridize to encoding sequences of a selected platelet VDCC α_1 subunit gene. In these aspects, probes of an appropriate length are prepared based on a consideration of the encoding sequence for a polypeptide of this invention. The ability of such probes to specifically hybridize to other encoding sequences lends them particular utility in a variety of embodiments. Most importantly, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

To provide certain of the advantages in accordance with the invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes probe sequences that are complementary to or mimic at least a 14 to 40 or so long nucleotide stretch of a nucleic acid sequence of the present invention, such as a sequence shown in any of SEQ ID NOs:1, 3, 5-8, 28, and 29. A size of at least 14 nucleotides in length helps to ensure that the fragment is of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 14 bases in length are generally preferred, though, to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 14 to 20 nucleotides, or even longer where desired, such as 30, 40, 50, 60, 100, 200, 300, or 500 nucleotides or up to the full length of any of SEQ ID NOs:1, 3, 5-8, 28, and 29. Such fragments can be readily prepared by, for example, directly synthesizing the fragment by chemical synthesis, by application of nucleic acid amplification technology, such as the PCR technology of U.S. Pat. No. 4,683,202, herein incorporated by reference, or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, a nucleotide sequence of the present invention can be used for its ability to selectively form duplex molecules with complementary stretches

of the gene. Depending on the application envisioned, one employs varying conditions of hybridization to achieve varying degrees of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one typically employs relatively stringent conditions to form the hybrids. For example, one selects relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M salt (*e.g.* NaCl), including particularly 200mM salt, at temperatures of 50°C to 70°C, including particularly temperatures of about 55°C, about 60°C and about 65°C. Such conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, less stringent hybridization conditions are typically needed to allow formation of the heteroduplex; one of skill in the art will know how to adjust the hybridization conditions for optimizing particular procedures. For example, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated by one of skill in the art using known methods to carry out the desired function or experiment, without undue experimentation.

In another aspect, the present invention provides assay kits for detecting the presence of a polypeptide of the present invention in biological samples, where the kits comprise a first antibody capable of immunoreacting with the polypeptide. Preferably, the assay kits of the invention further comprise a second container containing a second antibody that immunoreacts with the first antibody. More preferably, the antibodies used in the assay kits of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably still, the first and second antibodies comprise an indicator, and, preferably, the indicator is a radioactive label or an enzyme.

The present invention also provides an assay kit for screening agents. Such a kit can contain a polypeptide of the present invention. The kit can additionally contain reagents for detecting an interaction between an agent and a polypeptide of the present invention.

In an alternative aspect, the present invention provides assay kits for detecting the presence, in biological samples, of a polynucleotide that encodes a polypeptide of the present invention, the kits comprising a first container that contains a second polynucleotide identical or complementary to a segment of at least 10 contiguous nucleotide bases of, as a preferred example, any of SEQ ID NOs:1, 3, 5-8, 28, and 29. In another embodiment, the present invention provides assay kits for detecting the presence, in a biological sample, of antibodies that specifically binds a polypeptide of the present invention, the kits comprising a platelet VDCC α_1 subunit polypeptide that immunoreacts with the antibodies.

F. Mapping, Polynucleotide and Polypeptide Screening

In another embodiment of the invention, the nucleic acid sequences which encode a platelet VDCC α_1 subunit polypeptide can also be used to generate hybridization probes which are useful for mapping naturally occurring genomic sequences and/or disease loci. The sequences can be mapped to a particular chromosome or to a specific region of the chromosome using well-known techniques. Such techniques include FISH, FACS, or artificial chromosome constructions, such as yeast artificial chromosomes, bacterial artificial chromosomes, bacterial P1 constructions or single chromosome cDNA libraries as reviewed in Price, C. M. (1993) *Blood Rev.* 7:127-134, and Trask, B. J. (1991) *Trends Genet.* 7:149-154.

F.1. Mapping

FISH (as described in Verma et al. (1988) *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York, N.Y.) can be correlated with other physical chromosome mapping techniques and genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of *Science* (265:1981f). Correlation between the location of the gene encoding a platelet VDCC α_1 subunit polypeptide on a physical chromosomal map and a specific disease, or predisposition to a specific disease, can help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention can be used to detect differences in gene sequences between normal, carrier, or affected individuals.

In situ hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers can be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, reveals associated markers also found in other mammals such as humans even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once the disease or syndrome has been crudely localized by genetic linkage to a particular genomic region, for example, ataxia-telangiectasia (A-T) to 11q22-23 (Gatti, R. A. et al. (1988) *Nature* 336:577-580), any sequences mapping to that area can represent associated or regulatory genes for further investigation. The nucleotide sequences of the present invention can thus also be used to detect differences in the chromosomal location due to translocation, inversion, etc. among normal, carrier, or affected individuals.

The mapping methods of the present invention also employ genomic clones of the exons of a platelet VDCC α_1 subunit gene. Sequences for a human platelet VDCC α_1 subunit polypeptide gene are set forth in SEQ ID NOs:1-6, 28, and 29. Thus, the present invention also provides genetic assays based on the genomic sequence of the human platelet VDCC α_1 subunit polypeptide genes. An intronic sequence flanking an individual exon encoding a platelet VDCC α_1 subunit polypeptide is employed in the design of oligonucleotide primers suitable for the mutation analysis of human genomic DNA. Thus, intronic primers can be used to screen for genetic variants by a number of PCR-based techniques, including single-strand conformation polymorphism (SSCP) analysis (Orita, M., et al. (1989) *Proc Natl Acad Sci USA* 86(8):2766-70), SSCP/heteroduplex analysis, enzyme mismatch cleavage, and direct sequence analysis of amplified exons (Kestila, M., et al. (1998) *Mol Cell* 1(4), 575-82; Yuan, B., et al. (1999) *Hum Mutat* 14(5):440-6). Similar techniques can be applied to putative 5'-regulatory regions, e.g. the putative promoters 5' of a platelet VDCC α_1 subunit gene.

F.2. Polynucleotide Screening

Automated methods can also be applied the large-scale characterization of single nucleotide polymorphisms (SNPs) (Brookes, A. J. (1999) *Gene* 234(2):177-186; Wang, D. G., et al. (1998) *Science* 280(5366):1077-82) within and near a platelet VDCC α_1 subunit gene. Once genetic variants have been detected in specific patient populations, the present invention provides assays to detect the mutation by methods such as allele-specific hybridization (Stoneking, M., et al. (1991) *Am J Hum Genet* 48(2):370-82), or restriction analysis of amplified genomic DNA containing the specific mutation. Again, these detection methods can be automated using existing technology (Wang, D. G., et al. (1998) *Science* 280(5366):1077-82). In the case of genetic disease or human phenotypes caused by repeat expansion (Lafreniere, R. G., et al. (1997) *Nat Genet* 15(3):298-302; Timchenko, L. T., and Caskey, C. T. (1996) *Faseb J* 10(14):1589-97, the invention provides an assay based on PCR of genomic DNA with oligonucleotide primers flanking the involved repeat.

As used herein and in the claims, the term "polymorphism" refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. A polymorphic marker is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%. A polymorphic locus can be as small as one base pair.

The provided nucleic acid molecules can be labeled according to any technique known in the art, such as with radiolabels, fluorescent labels, enzymatic labels, sequence tags, etc. Such molecules can be used as allele-specific oligonucleotide probes. Body samples can be tested to determine whether a platelet VDCC α_1 subunit gene contains a polymorphism. Suitable body samples for testing include those comprising DNA, RNA or protein obtained from biopsies, including bone marrow biopsies; or from blood.

In one embodiment of the invention two pairs of isolated oligonucleotide primers are provided. These sets of primers are optionally derived from a platelet VDCC α_1 subunit exon. The oligonucleotide primers are useful, for example, in detecting a polymorphism of a platelet VDCC α_1 subunit gene. The primers direct amplification of a target polynucleotide prior to sequencing. In

another embodiment of the invention isolated allele specific oligonucleotides (ASO) are provided. The allele specific oligonucleotides are also useful in detecting a polymorphism of a platelet VDCC α_1 subunit gene.

The terms "substantially complementary to" or "substantially the sequence of" refer to sequences which hybridize to the sequences provided (e.g. SEQ ID NOs:1, 3, 5-8, 28, and 29) under stringent conditions as disclosed herein and/or sequences having sufficient identity with any of SEQ ID NOs:1, 3, 5-8, 28, and 29, such that the allele specific oligonucleotides of the invention hybridize to the sequence. The term "isolated" as used herein includes oligonucleotides substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which they can be associated, such association being either in cellular material or in a synthesis medium. A "target polynucleotide" or "target nucleic acid" refers to the nucleic acid sequence of interest e.g., a platelet VDCC α_1 subunit-encoding polynucleotide. Other primers which can be used for primer hybridization are readily ascertainable to those of skill in the art based upon the disclosure herein.

The primers of the invention embrace oligonucleotides of sufficient length and appropriate sequence so as to provide initiation of polymerization on a significant number of nucleic acids in the polymorphic locus. Specifically, the term "primer" as used herein refers to a sequence comprising two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and more preferably more than eight and most preferably at least about 20 nucleotides of a platelet VDCC α_1 subunit exonic or intronic region as are disclosed herein. Such oligonucleotides are preferably between ten and thirty bases in length. Such oligonucleotides can optionally further comprises a detectable label.

Environmental conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization, such as DNA polymerase, and a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but can be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization. The exact length of primer will

depend on many factors, including temperature, buffer, and nucleotide composition. The oligonucleotide primer typically contains 12-20 or more nucleotides, although it can contain fewer nucleotides.

Primers of the invention are designed to be "substantially" complementary to each strand of the genomic locus to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands under conditions which allow the agent for polymerization to perform. In other words, the primers should have sufficient complementarity with the 5' and 3' sequences flanking the transition to hybridize therewith and permit amplification of the genomic locus.

Oligonucleotide primers of the invention are employed in the amplification method which is an enzymatic chain reaction that produces exponential quantities of polymorphic locus relative to the number of reaction steps involved. Typically, one primer is complementary to the negative (-) strand of the polymorphic locus and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed by extension with an enzyme, such as the large fragment of DNA polymerase I (Klenow) and nucleotides, results in newly synthesized + and - strands containing the target polymorphic locus sequence. Because these newly synthesized sequences are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (i.e., the target polymorphic locus sequence) defined by the primers. The product of the chain reaction is a discreet nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

The oligonucleotide primers of the invention can be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and can be synthesized as described by Beaucage et al., *Tetrahedron Letters* 22:1859-1862 (1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066.

Any nucleic acid specimen, in purified or non-purified form, can be utilized as the starting nucleic acid or acids, providing it contains, or is

suspected of containing, a nucleic acid sequence containing the polymorphic locus. Thus, the method can amplify, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA can be single stranded or double stranded. In the event that RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA would be utilized. In addition, a DNA-RNA hybrid which contains one strand of each can be utilized. A mixture of nucleic acids can also be employed, or the nucleic acids produced in a previous amplification reaction herein, using the same or different primers can be so utilized. The specific nucleic acid sequence to be amplified, i.e., the polymorphic locus, can be a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it can be a minor fraction of a complex mixture, such as contained in whole human DNA.

DNA utilized herein can be extracted from a body sample, such as blood, tissue material (e.g. bone marrow tissue), and the like by a variety of techniques such as that described by Maniatis et. al. in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y., p 280-281 (1982). If the extracted sample is impure, it can be treated before amplification with an amount of a reagent effective to open the cells, or animal cell membranes of the sample, and to expose and/or separate the strand(s) of the nucleic acid(s). This lysing and nucleic acid denaturing step to expose and separate the strands will allow amplification to occur much more readily.

The deoxyribonucleotide triphosphates dATP, dCTP, dGTP, and dTTP are added to the synthesis mixture, either separately or together with the primers, in adequate amounts and the resulting solution is heated to about 90-100°C from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period, the solution is allowed to cool, which is preferable for the primer hybridization. To the cooled mixture is added an appropriate agent for effecting the primer extension reaction (called herein "agent for polymerization"), and the reaction is allowed to occur under conditions known in the art. The agent for polymerization can also be added together with the other reagents if it is heat stable. This synthesis (or amplification) reaction can occur at room temperature

up to a temperature above which the agent for polymerization no longer functions. Thus, for example, if DNA polymerase is used as the agent, the temperature is generally no greater than about 40°C. Most conveniently the reaction occurs at room temperature.

The agent for polymerization can be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase, polymerase muteins, reverse transcriptase, other enzymes, including heat-stable enzymes (i.e., those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation), such as *Taq* polymerase. Suitable enzyme will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each polymorphic locus nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths.

The newly synthesized strand and its complementary nucleic acid strand will form a double-stranded molecule under hybridizing conditions described herein and this hybrid is used in subsequent steps of the method. In the next step, the newly synthesized double-stranded molecule is subjected to denaturing conditions using any of the procedures described above to provide single-stranded molecules.

The steps of denaturing, annealing, and extension product synthesis can be repeated as often as needed to amplify the target polymorphic locus nucleic acid sequence to the extent necessary for detection. The amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion. *PCR. A Practical Approach*, ILR Press, Eds. McPherson et al. (1992).

The amplification products can be detected by Southern blot analysis with or without using radioactive probes. In one such method, for example, a small sample of DNA containing a very low level of the nucleic acid sequence of the polymorphic locus is amplified, and analyzed via a Southern blotting technique or similarly, using dot blot analysis. The use of non-radioactive

probes or labels is facilitated by the high level of the amplified signal. Alternatively, probes used to detect the amplified products can be directly or indirectly detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the probe, or will be able to ascertain such, using routine experimentation.

Sequences amplified by the methods of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as dideoxy sequencing, PCR, oligomer restriction (Saiki et al., *Bio/Technology* 3:1008-1012 (1985), allele-specific oligonucleotide (ASO) probe analysis (Conner et al., *Proc. Natl. Acad. Sci. U.S.A.* 80:278 (1983), oligonucleotide ligation assays (OLAs) (Landgren et. al., *Science* 241:1007, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landgren et. al., *Science* 242:229-237 (1988)).

Preferably, the method of amplifying is by PCR, as described herein and in U.S. Pat. Nos. 4,683,195; 4,683,202; and 4,965,188 each of which is hereby incorporated by reference; and as is commonly used by those of ordinary skill in the art. Alternative methods of amplification have been described and can also be employed as long as a VDCC locus amplified by PCR using primers of the invention is similarly amplified by the alternative means. Such alternative amplification systems include but are not limited to self-sustained sequence replication, which begins with a short sequence of RNA of interest and a T7 promoter. Reverse transcriptase copies the RNA into cDNA and degrades the RNA, followed by reverse transcriptase polymerizing a second strand of DNA.

Another nucleic acid amplification technique is nucleic acid sequence-based amplification (NASBA™) which uses reverse transcription and T7 RNA polymerase and incorporates two primers to target its cycling scheme. NASBA™ amplification can begin with either DNA or RNA and finish with either, and amplifies to about 10^8 copies within 60 to 90 minutes.

Alternatively, nucleic acid can be amplified by ligation activated transcription (LAT). LAT works from a single-stranded template with a single

primer that is partially single-stranded and partially double-stranded. Amplification is initiated by ligating a cDNA to the promoter oligonucleotide and within a few hours, amplification is about 10^8 to about 10^9 fold. The QB replicase system can be utilized by attaching an RNA sequence called MDV-1 to RNA complementary to a DNA sequence of interest. Upon mixing with a sample, the hybrid RNA finds its complement among the specimen's mRNAs and binds, activating the replicase to copy the tag-along sequence of interest.

Another nucleic acid amplification technique, ligase chain reaction (LCR), works by using two differently labeled halves of a sequence of interest which are covalently bonded by ligase in the presence of the contiguous sequence in a sample, forming a new target. The repair chain reaction (RCR) nucleic acid amplification technique uses two complementary and target-specific oligonucleotide probe pairs, thermostable polymerase and ligase, and DNA nucleotides to geometrically amplify targeted sequences. A 2-base gap separates the oligo probe pairs, and the RCR fills and joins the gap, mimicking normal DNA repair.

Nucleic acid amplification by strand displacement activation (SDA) utilizes a short primer containing a recognition site for *HincII* with short overhang on the 5' end which binds to target DNA. A DNA polymerase fills in the part of the primer opposite the overhang with sulfur-containing adenine analogs. *HincII* is added but only cuts the unmodified DNA strand. A DNA polymerase that lacks 5' exonuclease activity enters at the cite of the nick and begins to polymerize, displacing the initial primer strand downstream and building a new one which serves as more primer.

SDA produces greater than about a 10^7-fold amplification in 2 hours at 37°C. Unlike PCR and LCR, SDA does not require instrumented temperature cycling. Another amplification system useful in the method of the invention is the QB Replicase System. Although PCR is the preferred method of amplification of the invention, these other methods can also be used to amplify a platelet VDCC α_1 subunit locus as described herein. Thus, the term "amplification technique" as used herein and in the claims is meant to encompass all the foregoing methods.

In another embodiment of the invention a method is provided for identifying a subject having a polymorphism of a platelet VDCC α_1 subunit gene, comprising sequencing a target nucleic acid of a sample from a subject by dideoxy sequencing, preferably following amplification of the target nucleic acid.

In another embodiment of the invention a method is provided for identifying a subject having a polymorphism of a platelet VDCC α_1 subunit gene, comprising contacting a target nucleic acid of a sample from a subject with a reagent that detects the presence of a platelet VDCC α_1 subunit polymorphism and detecting the reagent. A number of hybridization methods and conditions are well known to those skilled in the art and are disclosed herein. Many of them are useful in carrying out the invention.

Accordingly, a nucleotide sequence of the present invention can be used for its ability to selectively form duplex molecules with complementary stretches of a platelet VDCC α_1 subunit gene. Depending on the application envisioned, one employs varying conditions of hybridization to achieve varying degrees of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one typically employs relatively stringent conditions to form the hybrids. For example, one selects relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M salt at temperatures of about 50°C to about 70°C including particularly temperatures of about 55°C, about 60°C and about 65°C. Such conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate polypeptide coding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions are typically needed to allow formation of the heteroduplex. Under such circumstances, one employs conditions such as 0.15M-0.9M salt, at temperatures ranging from about 20°C to about 55°C, including particularly temperatures of about 25°C, about 37°C, about 45°C, and about 50°C. Cross-hybridizing species can thereby be readily identified as positively

hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it is advantageous to employ a nucleic acid sequence of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator reagents are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one likely employs an enzyme tag such a urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, calorimetric indicator substrates are known which can be employed to provide a reagent visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein are useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the sample containing test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions depend *inter alia* on the particular circumstances based on the particular criteria required (depending, for example, on the G+ C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

The materials for use in the method of the invention are ideally suited for the preparation of a screening kit. Such a kit can comprise a carrier having compartments to receive in close confinement one or more containers such as vials, tubes, and the like, each of the containers comprising one of the separate

elements to be used in the method. For example, one of the containers can comprise an amplifying reagent for amplifying a platelet VDCC α_1 subunit DNA, such as the necessary enzyme(s) and oligonucleotide primers for amplifying target DNA from the subject.

The oligonucleotide primers include primers having a sequence derived from the group including, but not limited to: SEQ ID NOs:1, 3, 5-8, 28, and 29, or primer sequences substantially complementary or substantially homologous thereto. Oligonucleotide primers comprising target flanking 5' and 3' polynucleotide sequence have substantially the sequence set forth in the flanking 5' and 3' portions of any of SEQ ID NOs:1, 3, 5-8, 28, and 29, and sequences substantially complementary or homologous thereto. Other oligonucleotide primers for amplifying a platelet VDCC α_1 subunit will be known or readily ascertainable to those of skill in the art given the disclosure of the present invention presented herein.

A kit in accordance with the present invention can further comprise solutions, buffers or other reagents for extracting a nucleic acid sample from a biological sample obtained from a subject. Any such reagents as would be readily apparent to one of ordinary skill in the art fall within the scope of the present invention. By way of particular example, a suitable lysis buffer for the tissue or cells along with a suspension of glass beads for capturing the nucleic acid sample and an elution buffer for eluting the nucleic acid sample off of the glass beads comprise a reagent for extracting a nucleic acid sample from a biological sample obtained from a subject.

Other examples include commercially available extraction kits, such as the GENOMIC ISOLATION KIT A.S.A.P.™ (Boehringer Mannheim, Indianapolis, Indiana), Genomic DNA Isolation System (GIBCO BRL, Gaithersburg, Maryland), ELU-QUIK™ DNA Purification Kit (Schleicher & Schuell, Keene, New Hampshire), DNA Extraction Kit (Stratagene, La Jolla, California), TURBOGEN™ Isolation Kit (Invitrogen, San Diego, California), and the like. Use of these kits according to the manufacturer's instructions is generally acceptable for purification of DNA prior to practicing the methods of the present invention.

F.3. Polypeptide Screening

A method of screening for a disorder affecting calcium homeostasis in platelets is also provided. The method comprises: (a) obtaining a biological sample from a subject; (b) determining an amount of a platelet VDCC α_1 subunit polypeptide present in the biological sample; (c) determining the activity of a platelet VDCC α_1 subunit polypeptide present in the biological sample; and (d) detecting variations in calcium transport activity between a wild type platelet VDCC α_1 subunit polypeptide and an isolated platelet VDCC α_1 subunit polypeptide, any calcium transport activity variations between the wild type platelet VDCC α_1 subunit polypeptide and the isolated platelet VDCC α_1 subunit indicating the possibility of a disorder affecting calcium homeostasis in platelets. Detecting an amount of a platelet VDCC α_1 subunit polypeptide present in the biological sample can also be carried out in assessing a response to medication in a subject.

The nucleic acid sequences shown in SEQ ID NOs:1, 3, 5-8, 28, and 29 comprise platelet VDCC α_1 subunit polypeptide encoding sequences that are isolated from wild type cells. The sequence represents the platelet VDCC α_1 subunit nucleic acid sequence occurring in nature and existing without mutation. Therefore, wild type cells, as referred to herein, are those cells occurring in nature that contain non-mutated platelet VDCC α_1 subunit nucleic acid sequences. The wild type sequence is the native nucleic acid sequence and is the sequence against which assessments of polymorphism and mutation are made.

In another embodiment, the present invention provides an antibody that specifically binds a platelet VDCC α_1 subunit polypeptide. Preferably, an antibody of the invention is a monoclonal antibody. More preferred antibodies distinguish between a wild type form and a mutant or polymorphic form of a platelet VDCC α_1 subunit polypeptide. Techniques for preparing such antibodies are disclosed herein. The antibodies can be used to screen for the presence of a mutant or polymorphic form of a platelet VDCC α_1 subunit polypeptide in a manner analogous to that set forth above with respect to polynucleotide screening

G. Screening for Modulators of VDCC Biological Activity

In yet another aspect, the present invention provides a method of screening substances for their ability to affect or modulate the biological activity of platelet VDCC α_1 subunit gene products, and for their ability to affect or modulate *in vivo* platelet VDCC α_1 subunit levels. This modulation can affect platelet activation and other biological functions of platelets. Compounds identified via the screening methods of the present invention have application as anti-thrombotic agents or as agents for modulation of other biological events mediated by platelets.

Utilizing the methods and compositions of the present invention, screening assays for the testing of candidate substances are performed. A candidate substance is a substance which potentially can promote or inhibit the biological activity of gene product by binding or other intermolecular interaction with a platelet VDCC α_1 subunit gene or gene product or control sequence.

G.1. Method of Screening for Modulators of Platelet VDCC α_1 Subunit Biological Activity

A representative method of screening candidate substances for their ability to modulate platelet VDCC α_1 subunit biological activity comprises: (a) establishing replicate test and control samples that comprise a biologically active platelet VDCC α_1 subunit polypeptide; (b) administering a candidate substance to test samples; (c) measuring the biological activity of the platelet VDCC α_1 subunit polypeptide in the test and the control samples; and (d) determining whether the candidate substance modulates platelet VDCC α_1 subunit biological activity relative to an appropriate control. By "modulate" it is intended an increase, decrease, preservation, maintenance or other effect of any or all biological activities or properties of a platelet VDCC. By way of additional example, a candidate a candidate substance identified according to the screening assay described herein has an ability to facilitate preservation of stored platelets. Thus, a candidate substance identified according to the screening assay described herein has an ability to modulate platelet VDCC α_1 subunit biological activity.

Such a candidate compound has utility in the treatment of disorders and conditions associated with the biological activity of a platelet VDCC α_1 subunit.

Candidate compounds are typically about 500-1,000 daltons, and can be hydrophobic, polycyclic, or both, molecules. Such compounds should be considered as candidates for therapeutic intervention in accordance with the methods described herein below. Thus, compounds identified via the screening methods of the present invention have application as anti-thrombotic agents or as agents for modulation of other biological events mediated by platelets. Dosages of test agents can be determined by deriving dose-response curves, such as those disclosed in U.S. Patent No. 5,849,578, herein incorporated by reference.

In a cell-free system, the method comprises establishing a control system comprising a platelet VDCC α_1 subunit polypeptide and a ligand to which the platelet VDCC α_1 subunit polypeptide is capable of binding, establishing a test system comprising the platelet VDCC α_1 subunit polypeptide, the ligand, and a candidate compound, and determining whether the candidate compound modulates platelet VDCC α_1 subunit activity in a cell-free system. A representative ligand comprises a monoclonal antibody, and in this embodiment, the biological activity or property screened includes binding affinity. Additionally, the platelet VDCC α_1 subunit polypeptide can be provided in a lipid bi-layer in accordance with techniques disclosed by Malouf, N. N., et al., *Proc. Natl. Acad. Sci. USA* 84:5019-5023 (1987) to facilitate the simulation of *in vivo* conditions in a cell-free setting.

In another embodiment of the invention, a platelet VDCC α_1 subunit polypeptide (e.g., platelet VDCC α_1S subunit or platelet VDCC α_1D subunit) or catalytic or immunogenic fragment or oligopeptide thereof, can be used for screening libraries of compounds in any of a variety of drug screening techniques. The fragment employed in such screening can be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of binding complexes, between the platelet VDCC α_1 subunit polypeptide and the agent being tested, can be measured.

Another technique for drug screening which can be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application WO 84/03564, herein incorporated by reference. In this method, as applied to a platelet VDCC

α_1 subunit polypeptide, large numbers of different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with a platelet VDCC α_1 subunit polypeptide, or fragments thereof, and washed. Bound platelet VDCC α_1 subunit polypeptide is then detected by methods well known in the art. Purified platelet VDCC α_1 subunit polypeptide can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

As is well known in the art, a screening assay can provide a cell under conditions suitable for testing the modulation of VDCC biological activity and/or levels of calcium channels. These conditions include but are not limited to pH, temperature, tonicity, the presence of relevant metabolic factors (*e.g.*, metal ions such as for example Ca^{++}, growth factor, interleukins, or colony stimulating factors), and relevant modifications to the polypeptide such as glycosylation or prenylation. A polypeptide of the present invention can be expressed and utilized in a prokaryotic or eukaryotic cell. The host cell can also be fractionated into sub-cellular fractions where the receptor can be found. For example, cells expressing the polypeptide can be fractionated into the nuclei, the endoplasmic reticulum, vesicles, or the membrane surfaces of the cell. U.S. Patent Nos. 5,837,479; 5,645,999; 5,786,152; 5,739,278; and 5,352,660 also describe exemplary screening assays, and the entire contents of each are herein incorporated by reference.

In one embodiment, a screening assay is designed to be capable of discriminating candidate substances having selective ability to interact with or modulate one or more of the genes or gene products of the present invention but which substances are without a substantially overlapping activity with another gene or gene product. For example, a substance can modulate the biological activity of a platelet VDCC α_1S subunit but have no effect, or a diminished effect, on a platelet VDCC α_1D subunit. Such selective effect can comprise a 30% greater effect on one test sample versus another, or more preferably 100% or greater effect.

A method of identifying modulators of a platelet calcium channel polypeptide by rational drug design is provided in accordance with the present invention. The method comprises the steps of designing a potential modulator for a platelet calcium channel polypeptide that will form non-covalent bonds with amino acids in the substrate binding site based upon the structure of a platelet VDCC α_1 subunit polypeptide; synthesizing the modulator; and determining whether the potential modulator modulates the activity of a calcium channel. Modulators can be synthesized using techniques known in the art. The determination of whether the modulator modulates the biological activity of a calcium channel is made in accordance with the screening methods disclosed herein, or by other screening methods known in the art.

A screening assay of the present invention can also involve determining the ability of a candidate substance to modulate, *i.e.* preserve, inhibit or promote platelet VDCC α_1 subunit biological activity and preferably, to thereby modulate the biological activity of calcium channels in target cells. Target cells can be either naturally occurring cells known to contain a polypeptide of the present invention or transformed cells produced in accordance with a method of transformation set forth herein above. The test samples can further comprise a cell or cell line that expresses a platelet VDCC α_1 subunit polypeptide; the present invention also provides a recombinant cell line suitable for use in the exemplary method. Such cell lines can be mammalian, or human, or they can from another organism, including but not limited to yeast. Exemplary assays including genetic screening assays and molecular biology screens such as a yeast two-hybrid screen that will effectively identify platelet VDCC α_1 subunit-interacting genes important for calcium transport or other platelet VDCC α_1 subunit-mediated cellular method. One version of the yeast two-hybrid system has been described (Chien *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88:9578-9582) and is commercially available from Clontech (Palo Alto, California).

G.2. Assays for Biological Activity

Biological activity of a platelet VDCC α_1 subunit polypeptide can be determined, for example, by an assay disclosed immediately below. Indeed, the biological activity of a platelet VDCC α_1 subunit polypeptide of the present

invention can also be monitored as an indicator of drug therapy, such anti-thrombotic therapy or other therapy directed at platelet activity.

G.2.a. Measurement of Cytoplasmic Calcium Signal

The conduction of calcium by the VDCC is measured *in vitro* by measuring changes in cytoplasmic free (ionized) calcium concentrations. Cytoplasmic calcium concentrations are measured by performing flow cytometric analysis with the calcium indicators fluo-3 and fura red as detailed by Novak and Rabinovitch (1994) *Cytometry* 17:135-141. Briefly, platelets in whole blood, platelet rich plasma or biological buffers are incubated with esters of fluo-3 and fura red, and then aspirated into the flow cytometric instrument for measurement of calcium-dependent fluorescent changes. Cytoplasmic calcium concentrations are calculated from the ratio or fluo-3 and fura red relative fluorescence intensities. Experiments are performed with increasing doses of VDCC inhibitors with platelets that are in the resting state and subjected to different concentrations of activation agonists and VDCC antagonists and agonists.

G.2.b. Platelet Activation Reactions

The following methods are used to analyze the effect of VDCC channel inhibition, potentiation and/or structural manipulation on platelet function.

1. Activation time courses. Platelets are incubated at 37°C with [^{3}H]serotonin and $^{32}PO_4$ for one hour to respectively load dense granules with a secretion marker and label the cytoplasmic ATP pool for protein phosphorylation studies. During this time period, some samples are incubated with increasing concentrations of PGI_2 to elevate intracellular cAMP. The cells are exposed with agitation to different concentrations of thrombin, ADP, epinephrine, collagen, phorbol ester and A23187. Samples are withdrawn, quenched, and analyzed to characterize the time course of aggregation, secretion, morphological changes and clot retraction.

2. Aggregation. Aggregation is followed with flow cytometry by analyzing forward and side scatter patterns. Samples are be examined with light microscopy and scored morphometrically and subjected to optical aggrenometry.

3. Secretion. Platelets are diluted and centrifuged during the activation time course to obtain a supernatant with granule contents. Dense, alpha and lysozomal granule secretion are analyzed respectively by measuring [^{3}H]serotonin, thrombospondin and beta-N-acetylglucose aminadase levels in the supernatants. P-selectin expression on the surface membrane is quantified with flow cytometry after incubation of the cells with FITC-labeled monoclonal antibody to P-selectin (e.g., anti-P-selectin, Sigma Chemical Corp, St. Louis, MO).

4. Ultrastructural analysis. Platelet samples are subjected to scanning and transmission electron-microscopy to study shape change, pseudopodia extension, granule centralization, microfilament organization and microtubule structure.

5. Clot retraction. Microscale clot retraction analysis is performed in platelet-free plasma in siliconized glass capillary tubes with 10 μL samples. Time courses are initiated by adding thrombin for 1 unit/mL and calcium to 10 mM. The time course of retraction is followed by photographing the capillary sample every 10 seconds, and then the size of the clots are measured morphometrically.

6. Intracellular stimulus response coupling. Protein kinase activities are followed by performing 2D isoelectric focusing, SDS-PAG electrophoresis and autoradiography as we have detailed elsewhere (Fischer et al., 2000; White et al., 1990). The identity of tyrosine, threonine and serine protein kinase substrates is confirmed with Western analysis.

7. Analysis of cytoplasmic nucleotide pool. Platelets are incubated with ^{32}Pi to incorporate a radiolabel into cytoplasmic nucleotides. Cytoplasmic levels of ATP and ADP are measured with polyethyleneimine thin layer chromatography (TLC) and autoradiography as we have detailed elsewhere (Fischer et al., 2000). Ethanol solubility is used to determine the ratio of free vs. protein bound nucleotides (Holmsen, 1972). The intracellular nucleotide pool are examined before cross-linking, after cross-linking, and after lyophilization and rehydration. TLC analysis and sample preparation follow the procedure of Crabtree and Henderson (1971). Briefly, TLC plates are developed with 2 M sodium formate, pH = 3.4, and then subjected to autoradiography. ATP, ADP

and monophosphate spots are scraped and subjected to liquid scintillation counting to measure cytoplasmic levels of the nucleotides.

8. GPIIb-IIIa function: fibrinogen binding and trafficking. The density of unligated GPIIb-IIIa on the platelet surface is determined by measuring RGD inhabitable ^{125}I-fibrinogen binding as detailed by Sanders et al., (1996). Also, the amount of fibrinogen that is present on the surface of platelets from alpha-granule secretion is measured by quantifying FITC conjugated anti-fibrinogen monoclonal antibody binding with fluorescence. The ^{125}I-fibrinogen and anti-fibrinogen monoclonal antibody-FITC binding studies are conducted with standards so as to respectively yield the number of unoccupied and fibrinogen-ligated GPIIb-IIIa receptor per cell. The functionality of clathrin-dependent trafficking of surface bound fibrinogen to alpha-granules and clathrin-independent internalization to lyososomes (see, for example, Benke, 1992) is followed with confocal microscopy as described by Merricks et al. (1998).

9. GPIb-IX function: von Willebrand binding. The function of the von Willebrand receptor complex is analyzed by performing binding studies with ^{125}I-labeled von Willebrand factor (vWf) as detailed elsewhere (Khandelwal et al., 1997). Briefly, platelets are incubated with the radio labeled ligand, washed and then subjected to liquid scintillation counting. Binding studies are performed in the presence and absence of an inhibitory anti-GPIb monoclonal antibody (GPIb-mAb, Immunotech, Inc., Westbrook, Maine) and/or ristocetin.

G.3. Method of Screening for Modulators of *In Vivo* Platelet VDCC α_1 Subunit Levels

In accordance with the present invention there are also provided methods for screening candidate compounds for the ability to modulate *in vivo* platelet VDCC α_1 subunit levels and/or activity. Representative modulators of platelet VDCC α_1 subunit levels can comprise modulators of platelet VDCC α_1 subunit transcription or expression. Pharmaceuticals that increase or decrease the transcription or expression of platelet VDCC α_1 subunit encoding genes have important clinical application for the modulation of the biological activity of calcium channels. This modulation can affect calcium homeostasis in platelets.

This invention thus includes a method for discovery of compounds that modulate the expression levels of platelet VDCC α_1 subunit encoding genes, including not only the platelet VDCC α_1 subunit genes of the present invention but also other calcium channel polypeptide-encoding genes, and describes the use of such compounds. The general approach is to screen compound libraries for substances which increase or decrease expression of platelet VDCC α_1 subunit-encoding genes. Exemplary techniques are described in U.S. Patent Nos. 5,846,720 and 5,580,722, the entire contents of each of which are herein incorporated by reference.

While the following terms are believed to be well understood by one of skill in the art, the following definitions are set forth to facilitate explanation of the invention.

"Transcription" means a cellular method involving the interaction of an RNA polymerase with a gene that directs the expression as RNA of the structural information present in the coding sequences of the gene. The method includes, but is not limited to the following steps: (a) the transcription initiation, (b) transcript elongation, (c) transcript splicing, (d) transcript capping, (e) transcript termination, (f) transcript polyadenylation, (g) nuclear export of the transcript, (h) transcript editing, and (i) stabilizing the transcript. "Expression" generally refers to the cellular methodes by which a biologically active polypeptide is produced from RNA.

"Transcription factor" means a cytoplasmic or nuclear protein which binds to such gene, or binds to an RNA transcript of such gene, or binds to another protein which binds to such gene or such RNA transcript or another protein which in turn binds to such gene or such RNA transcript, so as to thereby modulate expression of the gene. Such modulation can additionally be achieved by other mechanisms; the essence of "transcription factor for a gene" is that the level of transcription of the gene is altered in some way.

In accordance with the present invention there is provided a method of identifying a candidate compound or molecule that is capable of modulating the transcription level of a gene encoding a platelet VDCC α_1 subunit polypeptide and thus is capable of acting as a therapeutic agent in the modulation of platelet VDCC α_1 subunit polypeptide effects. This modulation can affect

calcium homeostasis in platelets, platelet activation and other biological functions of platelets and can also effect platelet storage or production of platelet products. Such modulation can be direct, i.e., through binding of a candidate molecule directly to the nucleotide sequence, whether DNA or RNA transcript, or such modulation can be achieved via one or more intermediaries, such as proteins other than a platelet VDCC α_1 subunit polypeptide which are affected by the candidate compound and ultimately modulate platelet VDCC α_1 subunit polypeptide transcription by any mechanism, including direct binding, phosphorylation or dephosphorylation, etc.

This method comprises contacting a cell or nucleic acid sample with a candidate compound or molecule to be tested. These samples contain nucleic acids which can contain elements that modulate transcription and/or translation of a platelet VDCC α_1 subunit gene, such as a platelet VDCC α_1 subunit promoter or putative upstream regulatory region, or other VDCC α_1 subunit promoter or putative upstream regulatory region, and a DNA sequence encoding a polypeptide which can be detected in some way. Thus, the polypeptide can be described as a "reporter" or "marker." Preferably, the candidate compound directly and specifically transcriptionally modulates expression of the platelet VDCC α_1 subunit polypeptide-encoding gene. Such have therapeutic or pharmaceutical uses in treating platelet VDCC α_1 subunit polypeptide-related diseases and/or disorders, in platelet-based medicine and laboratory efforts, and in preserving and transporting platelets.

The DNA sequence is coupled to and under the control of the promoter, under conditions such that the candidate compound or molecule, if capable of acting as a transcriptional modulator of a gene encoding platelet VDCC α_1 subunit polypeptide, causes the polypeptide to be expressed and so produces a detectable signal, which can be assayed quantitatively and compared to an appropriate control. Candidate compounds or molecules of interest can include those which increase or decrease, *i.e.*, modulate, transcription from a platelet VDCC α_1 subunit promoters. The reporter gene can encode a reporter known in the art, such as luciferase, or it can encode a platelet VDCC α_1 subunit.

In certain embodiments of the invention the polypeptide so produced is capable of complexing with an antibody or is capable of complexing with biotin.

In this case the resulting complexes can be detected by methods known in the art. The detectable signal of this assay can also be provided by messenger RNA produced by transcription of said reporter gene. Exactly how the signal is produced and detected can vary and is not the subject of the present invention; rather, the present invention provides the nucleotide sequences and/or putative regulatory regions of a platelet VDCC α_1 subunit for use in such an assay. The molecule to be tested in these methods can be a purified molecule, a homogenous sample, or a mixture of molecules or compounds. Further, in the method of the invention, the DNA in the cell can comprise more than one modulatable transcriptional regulatory sequence.

In accordance with the present invention there is also provided a rapid and high throughput screening method that relies on the methods described above. This screening method comprises separately contacting each of a plurality of substantially identical samples. In such a screening method the plurality of samples preferably comprises more than about 10^4 samples, or more preferably comprises more than about 5×10^4 samples.

G.4. Animal Models

In addition, animal-based systems can be used to identify compounds capable of modulating platelet VDCC α_1 subunit biological activity. Such animal models can be used for the identification of drugs, pharmaceuticals, therapies, and interventions that can be effective in modulating platelet VDCC α_1 subunit polypeptide biological activity. For example, animal models can be exposed to a compound that is suspected of exhibiting an ability to modulate platelet VDCC α_1 subunit polypeptide biological activity at a sufficient concentration and for a time sufficient to elicit such modulation of platelet VDCC α_1 subunit polypeptide biological activity in the exposed animals. The response of the animals to the exposure can be monitored by assessing *in vivo* platelet VDCC α_1 subunit polypeptide expression levels and activity, or by testing biological samples from the animal. As in the methods described above, the mechanism by which a compound modulates a platelet VDCC α_1 subunit polypeptide activity or achieves therapeutic effects can vary; the utility of the present invention does not depend on the precise mechanism by which an effect is achieved.

For example, an animal model of the present invention can comprise a pig with targeted modification of a pig platelet VDCC α_1 subunit polypeptide genes, as described herein above.

H. Modulation of Platelet VDCC α_1 Subunit Biological Activity in a Laboratory or Clinical Setting

An aspect of the invention encompasses any treatments that alter any aspect of platelet VDCC α_1 subunit polypeptide biological activity. Such methods of modulating the biological activity of a platelet calcium channel polypeptide are applicable in the laboratory and/or clinical setting to enhance the capability to store, freeze dry, dehydrate or otherwise manipulate platelets or platelet products, as well as being applicable in therapeutic intervention in a subject. Therapeutic intervention in a subject can encompass, for example, anti-thrombotic therapy or therapy via modulation of other biological events mediated by platelets. Representative anti-thrombotic therapy comprises treatment or prevention of arterial thromboses, e.g. the blood clots that cause heart attacks and strokes. Another representative therapeutic application comprises increasing platelet function in bleeding disorders (e.g. hemophilia), whether acquired or inherited.

As used herein, the terms "activity" and "biological activity" are meant to be synonymous and are meant to refer to any biological activity of a platelet VDCC α_1 subunit polypeptide (e.g., platelet VDCC α_1S subunit or platelet VDCC α_1D subunit). Representative biological activities of a platelet VDCC α_1 subunit include calcium transport or other biological activity in accordance with the present invention.

With respect to the therapeutic methods of the present invention, a preferred subject is a vertebrate subject. A preferred vertebrate is warm-blooded; a preferred warm-blooded vertebrate is a mammal. A preferred mammal is a pig or, most preferably, a human. As used herein and in the claims, the term "patient" is contemplated to include both human and animal patients. Thus, veterinary diagnostic and therapeutic uses are provided in accordance with the present invention and comprise a preferred embodiment of the present invention.

Contemplated is the treatment of mammals such as humans, as well as those mammals of importance due to being endangered, such as Siberian tigers; of economical importance, such as animals raised on farms for consumption by humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos. Examples of such animals include but are not limited to: carnivores such as cats and dogs; swine, including pigs, hogs, and wild boars; ruminants and/or ungulates such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels; and horses. Also contemplated is the treatment of birds, including the treatment of those kinds of birds that are endangered and/or kept in zoos, as well as fowl, and more particularly domesticated fowl, *i.e.*, poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economical importance to humans. Thus, contemplated is the treatment of livestock, including, but not limited to, domesticated swine, ruminants, ungulates, horses, poultry, and the like.

H.1. Modulation of Platelet VDCC α_1 Subunit Polypeptide Biological Activity

In one embodiment, the present inventive method comprises administering to a cell a substance that modulates, *i.e.*, inhibits or promotes a biological activity of a platelet VDCC α_1 subunit polypeptide. Such a substance can be identified according to any of the screening assays set forth above, either *in vitro* or *in vivo*. Representative cells include platelets and megakaryocytes. The cell can be in an *in vitro* setting or can be in a subject to be treated, such as a warm-blooded vertebrate as described herein above.

The method comprises treating a vertebrate subject suffering from a disorder associated with or mediated by platelet VDCC α_1 subunit polypeptide biological activity by administering to the subject an effective amount of a substance identified according to a screening assay described above. By the term "modulating", it is meant that the substance can either promote or inhibit the biological activity of a platelet VDCC α_1 subunit, depending on the disorder to be treated, and can affect one or several of the platelet VDCC α_1 subunit polypeptides, including the platelet VDCC α_1D subunit polypeptide or the

platelet VDCC α_1S subunit polypeptide, as well as other ion transporters, or other unrelated genes or gene products.

Therapeutic treatment can comprise the administration of antibodies against a chosen region of a platelet VDCC α_1 subunit polypeptide, the administration of a protein that enhances activity, or the administration of a protein that inhibits the transcription of the platelet VDCC α_1 subunit polypeptide. Such administration can provide treatment of disorders which can be caused or exacerbated by platelet VDCC α_1 subunit polypeptide-mediated mechanisms.

Insofar as a modulator of platelet VDCC α_1 subunit polypeptide activity can take the form of a polypeptide or of an anti-platelet VDCC α_1 subunit polypeptide monoclonal antibody or fragment thereof, it is to be appreciated that the potency can vary, and therefore a "therapeutically effective" amount can vary. However, as shown by the present assay methods, one skilled in the art can readily assess the potency and efficacy of a candidate platelet VDCC α_1 subunit polypeptide biological activity modulator of this invention and adjust the therapeutic regimen accordingly. A modulator of platelet VDCC α_1 subunit biological activity can be evaluated by a variety of techniques, including through the use of a responsive reporter, which drives expression of a reporter gene; interaction of platelet VDCC α_1 subunit polypeptides with a monoclonal antibody as described herein; and other assays known in the art and described herein.

The monoclonal antibodies or polypeptides of the invention can be administered parenterally by injection or by gradual infusion over time. Although the tissue to be treated can typically be accessed in the body by systemic administration and therefore most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated where there is a likelihood that the tissue targeted contains the target molecule and are known to those of skill in the art. The compositions are formulated in an appropriate manner and administered in a manner compatible with the dosage formulation.

H.2. Monoclonal Antibodies

The present invention describes, in one embodiment, platelet VDCC α_1 subunit polypeptide modulators in the form of monoclonal antibodies which were elicited in response to platelet VDCC α_1 subunit but which can immunoreact with any platelet VDCC α_1 subunit polypeptide, or with a specific isoform of a platelet VDCC α_1 subunit polypeptide, and bind the platelet VDCC α_1 subunit polypeptide to modulate biological activity. The invention also describes cell lines that produce the antibodies, methods for producing the cell lines, and methods for producing the monoclonal antibodies.

The term "antibody" or "antibody molecule" refers collectively to a population of immunoglobulin molecules and/or immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain a paratope. A paratope is the portion or portions of an antibody that is or are responsible for that antibody binding to an antigenic determinant, or epitope.

Representative antibodies for use in the present invention are intact immunoglobulin molecules, substantially intact immunoglobulin molecules, single chain immunoglobulins or antibodies, those portions of an immunoglobulin molecule that contain the paratope, including antibody fragments. Indeed, it is within the scope of the present invention that a monovalent modulator can optionally be used. Thus, the terms "modulate", "modulating", and "modulator" are intended to encompass such a mechanism.

The term "monoclonal antibody" refers to a population of antibody molecules that contain only one species of paratope and thus typically display a single binding affinity for any particular epitope with which it immunoreacts; a monoclonal antibody can have a plurality of antibody combining sites, each immunospecific for a different epitope, *e.g.*, a bispecific monoclonal antibody. Methods of producing a monoclonal antibody, a hybridoma cell, or a hybridoma cell culture are described above.

It is also possible to determine, without undue experimentation, if a monoclonal antibody has the same or equivalent specificity or immunoreaction characteristics as a monoclonal antibody of this invention by ascertaining whether the former prevents the latter from binding to a preselected target molecule. If the monoclonal antibody being tested competes with the

monoclonal antibody of the invention, as shown by a decrease in binding by the monoclonal antibody of the invention in standard competition assays for binding to the target molecule when present in the solid phase, then it is likely that the two monoclonal antibodies bind to the same, or a closely related, epitope.

Still another way to determine whether a monoclonal antibody has the specificity of a monoclonal antibody of the invention is to pre-incubate the monoclonal antibody of the invention with the target molecule with which it is normally reactive, and then add the monoclonal antibody being tested to determine if the monoclonal antibody being tested is inhibited in its ability to bind the target molecule. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or functionally equivalent, epitopic specificity as the monoclonal antibody of the invention.

An additional way to determine whether a monoclonal antibody has the specificity of a monoclonal antibody of the invention is to determine the amino acid residue sequence of the CDR regions of the antibodies in question. "CDRs" (complementarity-determining regions) mean the three subregions of the light or heavy chain variable regions which have hypervariable sequences and form loop structures that are primarily responsible for making direct contact with antigen. Antibody molecules having identical, or functionally equivalent, amino acid residue sequences in their CDR regions have the same binding specificity. Methods for sequencing polypeptides are well known in the art. Further, other ways of determining whether antibodies have similar immunospecificities are known in the art and can be useful in practicing the present invention.

The immunospecificity of an antibody, its target molecule binding capacity, and the attendant affinity the antibody exhibits for the epitope are defined by the epitope with which the antibody immunoreacts. The epitope specificity is defined at least in part by the amino acid residue sequence of the variable region of the heavy chain of the immunoglobulin that comprises the antibody, and in part by the light-chain-variable-region amino acid residue sequence. Use of the terms "having the binding specificity of" or "having the binding preference of" indicates that equivalent monoclonal antibodies exhibit

the same or similar immunoreaction (binding) characteristics and compete for binding to a preselected target molecule.

Humanized monoclonal antibodies offer particular advantages over monoclonal antibodies derived from other mammals, particularly insofar as they can be used therapeutically in humans. Specifically, human antibodies are not cleared from the circulation as rapidly as "foreign" antigens, and do not activate the immune system in the same manner as foreign antigens and foreign antibodies. Methods of preparing "humanized" antibodies are generally well known in the art, and can readily be applied to the antibodies of the present invention.

The use of a molecular cloning approach to generate antibodies, particularly monoclonal antibodies, and more particularly single chain monoclonal antibodies, is also provided. The production of single chain antibodies has been described in the art, see *e.g.*, U.S. Patent No. 5,260,203, the contents of which are herein incorporated by reference. For this approach, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from the spleen of the immunized animal, and phagemids expressing appropriate antibodies are selected by panning on endothelial tissue. The advantages of this approach over conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination in a single chain, which further increases the chance of finding appropriate antibodies. Thus, an antibody of the present invention, or a "derivative" of an antibody of the present invention, pertains to a single polypeptide chain binding molecule which has binding specificity and affinity substantially similar to the binding specificity and affinity of the light and heavy chain aggregate variable region of an antibody described herein.

H.3. Other Modulators

Given the disclosure of the platelet VDCC α_1 subunit polypeptide activity in tissues herein, chemical compounds (*e.g.* small molecule mimetics) can be used to modulate platelet VDCC α_1 subunit polypeptide activity in tissues in accordance with the methods of the present invention. The identification of such compounds is facilitated by the description of screening assays directed

to platelet VDCC α_1 subunit polypeptide activity in tissues presented above. Such compounds are typically about 500-1,000 daltons, and can be hydrophobic, polycyclic, or both, molecules.

H.4. Gene Therapy

Platelet VDCC α_1 subunit polypeptide genes can be used for gene therapy in accordance with the present invention. Exemplary gene therapy methods, including liposomal transfection of nucleic acids into host cells, are described in U.S. Patent Nos. 5,279,833; 5,286,634; 5,399,346; 5,646,008; 5,651,964; 5,641,484; and 5,643,567, the contents of each of which are herein incorporated by reference.

Briefly, gene therapy directed toward modulation of platelet VDCC α_1 subunit polypeptide levels, to thereby affect or modulate the biological activity of platelet VDCC α_1 subunit polypeptide in a target cell is described. This modulation can affect calcium transport, to thereby affect platelet activation or other biological effect. In one embodiment, a therapeutic method of the present invention provides a method for modulation of platelet VDCC α_1 subunit polypeptide levels comprising the steps of: (a) delivering to the cell an effective amount of a DNA molecule comprising a polynucleotide that encodes a polypeptide that modulates the biological activity of one or more than one platelet VDCC α_1 subunit polypeptide; and (b) maintaining the cell under conditions sufficient for expression of said polypeptide.

In a preferred embodiment, the delivered polypeptide comprises the sequence of SEQ ID NO:2 or 4. Delivery can be accomplished by injecting the DNA molecule into the cell. Where the cell is in a subject, administering comprises: (a) providing a vehicle that contains the DNA molecule; and (b) administering the vehicle to the subject.

A vehicle is preferably a cell transformed or transfected with the DNA molecule or a transfected cell derived from such a transformed or transfected cell. An exemplary and preferred transformed or transfected cell is a lymphocyte or a tumor cell from the tumor being treated. Means for transforming or transfecting a cell with a DNA molecule of the present invention are set forth above.

Alternatively, the vehicle is a virus or an antibody that specifically infects or immunoreacts with an antigen of the target tissue or tumor. An advantage of a viral infection system is that it allows for a very high level of infection into the appropriate recipient cell. Also, antibodies have been used to target and deliver DNA molecules.

It is also envisioned that this embodiment of the present invention can be practiced using alternative viral or phage vectors, including retroviral vectors, adenoviral vectors and vaccinia viruses whose genome has been manipulated in alternative ways so as to render the virus non-pathogenic. Methods for creating such a viral mutation are set forth in detail in U.S. Pat. No. 4,769,331, incorporated herein by reference.

In a preferred embodiment, the vector is a recombinant vector comprising: (a) a sequence of genomic viral DNA showing affinity for host cells; (b) a DNA sequence encoding a platelet VDCC α_1 subunit polypeptide and operatively linked to said sequence of genomic viral DNA; and (c) a selectable marker.

H.5. Method of Modulating *In Vivo* Platelet VDCC α_1 subunit polypeptide Levels in the Treatment of Related Diseases and Disorders

A method for transcriptionally modulating in a cell or in a multicellular organism the expression of a gene encoding a platelet VDCC α_1 subunit polypeptide to modulate platelet VDCC α_1 subunit polypeptide biological activity in the cell or organism is also provided in accordance with the present invention. This method comprises administering to cell or to the organism a compound at a concentration effective to transcriptionally modulate expression of platelet VDCC α_1 subunit polypeptide or cotransporters. Representative cells include platelets and megakaryocytes. The cell can be in an *in vitro* setting or can be in an organism to be treated, such as a warm-blooded vertebrate as described herein above.

In accordance with the present invention, the provided compound can optionally comprise an antibody or polypeptide prepared as described above and which transcriptionally modulates expression of platelet VDCC α_1 subunit

polypeptides. Optionally, the antibody or polypeptide directly binds to DNA or RNA, or directly binds to a protein involved in transcription.

Particular chemical entities (*e.g.* small molecule mimetics) for use in accordance with the present invention do not naturally occur in any cell, whether of a multicellular or a unicellular organism. Even more particularly, the chemical entity is not a naturally occurring molecule, *e.g.* it is a chemically synthesized entity. Optionally, the compound can bind a modulatable transcription sequence of the gene. For example, the compound can bind a promoter region upstream of a nucleic acid sequence encoding a platelet VDCC α_1 subunit polypeptide.

In the methods above, modulation of transcription results in either upregulation or downregulation of expression of the gene encoding the protein of interest, depending on the identity of the molecule which contacts the cell.

H.6. Antisense Oligonucleotide Therapy

It is also provided according to the present invention that expression of a platelet VDCC α_1 subunit polypeptide can be modulated in a vertebrate subject through the administration of an antisense oligonucleotide derived from a nucleic acid molecule encoding a platelet VDCC α_1 subunit polypeptide, such as those described in SEQ ID NO:2 and 4. Therapeutic methods utilizing antisense oligonucleotides have been described in the art, for example, in U.S. Patent Nos. 5,627,158 and 5,734,033, the contents of each of which are herein incorporated by reference.

H.7. Dosages

As used herein, an "effective" dose refers to one that is administered in doses tailored to a particular application in which calcium transport modulation or other modulation of platelet VDCC α_1 subunit biological activity is sought. For example, after review of the disclosure herein of the present invention, one of ordinary skill in the art can tailor the dosages to an individual patient, taking into account the particular formulation and method of administration to be used with the composition as well as patient height, weight, severity of symptoms, and stage of the disorder to be treated.

An effective dose and a therapeutically effective dose are generally synonymous. However, compounds can be administered to patients having

reduced symptoms or even administered to patients as a preventative measure. Hence, the composition can be effective in therapeutic treatment even in the absence of symptoms of the disorder.

A unit dose can be administered, for example, 1 to 4 times per day. Most preferably, the unit dose is administered twice a day (BID). The dose depends on the route of administration and the formulation of a composition containing the compound or compounds. Further, it will be appreciated by one of ordinary skill in the art after receiving the disclosure of the present invention that it can be necessary to make routine adjustments or variations to the dosage depending on the combination of agents employed, on the age and weight of the patient, and on the severity of the condition to be treated.

Such adjustments or variations, as well as evaluation of when and how to make such adjustments or variations, are well known to those of ordinary skill in the art of medicine. Evaluation parameters and techniques can vary with the patient and the severity of the disease. Particularly useful evaluative techniques are disclosed in the Examples.

H.7.1. Gene Therapy Vector Construct Dosing.

Maximally tolerated dose (MTD) of vector construct when administered directly into the affected tissue is determined. Primary endpoints are: 1) the rate of transduction in abnormal and/or normal cells, 2) the presence and stability of this vector in the systemic circulation and in affected cells, and 3) the nature of the systemic (fever, myalgias) and local (infections, pain) toxicities induced by the vector. A secondary endpoint is the clinical efficacy of the vector construct.

For example, a 4 mL serum-free volume of viral (e.g. adenoviral, retroviral, etc.) vector construct (containing up to 5 $X10^7$ viral particles in AIM V media) is administered daily per session. During each session, 1 mL of medium containing the appropriate titer of vector construct is injected into 4 regions of the affected tissue for a total of 4 mL per session in a clinical examination room. This is repeated daily for 4 days (4 sessions). This 16 mL total inoculum volume over 4 days is proportionally well below the one safely tolerated by nude mice (0.5 mL/20 g body weight).

Patient evaluation includes history and physical examination prior to initiation of therapy and daily during the 4 day period of vector construct injection. Toxicity grading is done using the ECOG Common Toxicity Criteria. CBC, SMA-20, urinalysis, and conventional studies are performed daily during this period.

H.7.2. Dose escalation and MTD.

Patients are treated with 3 x 10^6 viral particles x 4. Once they have all recovered from all grade 2 or less toxicities (except alopecia), and as long as grade 3-4 toxicity is not encountered, a subsequent dose level is initiated in patients. As one grade 3 or 4 toxicity occurs at a given dose level, a minimum of 6 patients are enrolled at that level. As only 1 of 6 patients has grade 3 or 4 toxicity, dose escalation continues. The MTD of vector construct is defined as the dose where 2 of 6 patients experience grade 3 or 4 toxicity. If 2 of 3, or if 3 of 6 patients experience grade 3 or 4 toxicity, the MTD is defined as the immediately lower dose level.

The following escalation schema is followed: 1) level 1, 3 x 10^6 viral particles; 2) level 2, 1 x 10^7; 3) level 3, 3 x 10^7; 4) level 4, 5 X 10^7. Patients with measurable disease are evaluated for a clinical response to vector construct. Histology and local symptoms are followed.

H.8. Formulation of Therapeutic Compositions

The platelet VDCC α_1 subunit polypeptide biological activity modulating substances, gene therapy vectors, and substances that inhibit or promote expression of a platelet VDCC α_1 subunit polypeptide encoding nucleic acid segment described above are adapted for administration as a pharmaceutical compositions as described above. Additional formulation and dose preparation techniques have been described in the art, see for example, those described in U.S. Patent No. 5,326,902 issued to Seipp *et al.* on July 5, 1994, U.S. Patent No. 5,234,933 issued to Marnett *et al.* on August 10, 1993, and PCT Publication WO 93/25521 of Johnson *et al.* published December 23, 1993, the entire contents of each of which are herein incorporated by reference.

For the purposes described above, the identified substances can normally be administered systemically or partially, usually by oral or parenteral administration. The doses to be administered are determined depending upon

age, body weight, symptom, the desired therapeutic effect, the route of administration, and the duration of the treatment, etc.; one of skill in the art of therapeutic treatment will recognize appropriate procedures and techniques for determining the appropriate dosage regimen for effective therapy. Various compositions and forms of administration are provided and are generally known in the art. Other compositions for administration include liquids for external use, and endermic linaments (ointment, etc.), suppositories and pessaries which comprise one or more of the active substance(s) and can be prepared by known methods.

Thus, the present invention provides pharmaceutical compositions comprising a polypeptide, polynucleotide, or molecule or compound of the present invention and a physiologically acceptable carrier. More preferably, a pharmaceutical composition comprises a compound discovered via the screening methods described herein below.

A composition of the present invention is typically administered parenterally in dosage unit formulations containing standard, well-known nontoxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term "parenteral" as used herein includes intravenous, intra-muscular, intra-arterial injection, or infusion techniques.

Injectable preparations, for example sterile injectable aqueous or oleaginous suspensions, are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Preferred carriers include neutral saline solutions buffered with phosphate, lactate, Tris, and the like. Of course, one purifies the vector

sufficiently to render it essentially free of undesirable contaminants, such as defective interfering adenovirus particles or endotoxins and other pyrogens such that it does not cause any untoward reactions in the individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation or column chromatography.

A transfected cell can also serve as a carrier. By way of example, a liver cell can be removed from an organism, transfected with a polynucleotide of the present invention using methods set forth above and then the transfected cell returned to the organism (e.g. injected intra-vascularly).

EXAMPLES

The following Examples have been included to illustrate modes of the invention. Certain aspects of the following Examples are described in terms of techniques and procedures found or contemplated by the present inventors to work well in the practice of the invention. These Examples are exemplified through the use of standard laboratory practices of the inventors. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications and alterations can be employed without departing from the spirit and scope of the invention.

Example 1

Isolation of Fresh Platelets and Cultured Megakaryocytes

Due to the paucity of undegraded mRNA in stored human platelets, mRNA was isolated from platelet precursor cells, megakaryocytes, in culture. The findings disclosed herein were confirmed in freshly isolated porcine platelets. Freshly isolated human platelets were used for the ultrastructural studies.

Blood was collected in sodium citrate (9:1 v/v) from informed and consenting healthy volunteers and from pigs according to the National Institute of Health (NIH) human and animal utilization guidelines and approved by University of North Carolina-Chapel Hill (UNC-CH) committees. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 800 g for 5 min at room temperature (r. t.), and platelets were isolated at 2000 g centrifugation

for 15 min at r. t. The platelets were immediately used for mRNA or protein isolations.

Meg 01 cells (a human megakaryocytic cell line available from the American Type Culture Collection (ATCC) in Manassas, Virginia) were grown in RPMI (GIBCO BRL, Gaithersburg, Maryland) supplemented with 10% fetal calf serum in 10% CO_2 at 37°C. Cells were harvested by pelleting. Human megakaryocytes were obtained from discarded bone marrow smears with permission from the (UNC-CH) Internal Review Board (IRB) committee.

Example 2

Preparation of mRNA for Northern Blots and RT-PCR

Platelets or megakaryocytes were lysed in a reagent sold under the registered trademark TRIPURE® by Boeringer Manheim of Indianapolis, Indiana via repetitive pipetting. Total RNA was isolated according to the specifications supplied by the vendor. The total RNA was treated with 20 units of RNAase-free DNAase per 100 µg of total RNA. mRNA was enriched using a mRNA kit sold under the registered trademark OLIGOTEX® by QIAGEN GMBH of Hilden, Germany. Approximately 10 µg of mRNA was size fractionated on gels (Ambion Inc., Austin, Texas) in formaldehyde denaturing conditions for Northern blots. See Fig. 4. mRNA was transferred onto a membrane by capillary action for Northern blots.

Antisense riboprobes of 1033 nucleotides (nt) were synthesized to correspond to the region between IV S_3 and amino acid 1531 (aa1531) for α_1S and between IV S_3 and amino acid 1663 (aa1663) for α_1D (Seino S., Chen L., et al., *Proc Natl Acad Sci U S A.* (1992) 89:584-588). These probes were obtained from the PCR reaction using oligonucleotides 1x2 (Table 3). The probes were transcribed as the antisense strand from the linearized PCR-TOPO® cloning vector (Invitrogen Corporation of Carlsbad, California) that contained the PCR product as an insert.

First strand cDNA was synthesized from 0.2 µg mRNA with Oligo-dt or random hexamers as primers. A reverse transcriptase enzyme sold under the trademark SUPERSCRIPT II™ by Life Technologies, Inc. of Rockville, Maryland was used, and the reaction was carried out according to specifications provided by the vendor. Following the synthesis of the first

strand, the mRNA template was removed with RNAase treatment. The cDNA obtained was ethanol precipitated and then used for PCR amplification. PCR primers were designed based upon regions of VDCC α_1S (Hogan K., et al., *Genomics* (1994) 24:608-609) and α_1D (Seino S., et al., *Proc Natl Acad Sci U S A* (1992) 89:584-588) subunits, such that isoform specific intervening sequences containing regions previously reported to have functional importance would be amplified. Primers (Table 3) were used at a concentration of 100 picomoles per reaction.

PCR was carried out according to standard techniques. The PCR products were examined by agarose gel electrophoresis and cloned into a vector sold under the registered trademark PCR II-TOPO® by Invitrogen Corporation of Carlsbad, California. The clones were screened by PCR, and the desired cDNA was prepared using a DNA Miniprep kit (Qiagen, Inc. of Sorrento, California) for sequencing by the UNC-CH Automated DNA Sequencing Facility.

Table 3

Oligo-nucleotide No.	5' position	3' position	Oligonucleotide Combination for PCR amplification	ref.	Predicted polypeptide region amplified
1	3763	3785	1x2 (see Fig. 1)	α_1S	IV S_3 - aa1531
2	4796	4760	1x2 (see Fig. 1)	α_1D	IV S3 - aa1633
3	4684	4705	3x4	α_1S	aa1483 - end of the polypeptide
4	5845	5824			
5	6661	6633	3x5	α_1D	aa1586 - end of the polypeptide
6	3240	3265	6x7	α_1D	III P - IV P
7	4220	4194			
8	2221	2243	8x9	α_1S	Intracytoplasmic loop II - III
9	2622	2601			
10	2949	2972	10x11	α_1S	III S_4 - IV S_3

Oligonucleotide primers were used in PCR reactions to amplify regions of α_1S and α_1D from platelets and megakaryocytes. The 5' and 3' positions are numbered according to the cDNAs accessed in Gen Bank:L33798 (Hogan, K., et al. *Genomics* (1994) 24:608-609) and M83566 (Seino, S., et al., *Proc Natl Acad Sci U S A.* (1992) 89:584-588) for the human α_1S and α_1D cDNAs, respectively. The predicted polypeptide position of the encoded protein is designated. P = pore.

Example 3

Antibodies and Immunoprecipitation

A rabbit antibody was generated against the synthetic peptide NEELRAIIKKIWKRTSMKLL (SEQ ID NO:27) which corresponds to the sequence aa 1487-1506 (arrow in Fig. 1A) in the putative intracytoplasmic carboxyl-terminal region of adult rabbit and human α_1S (Tanabe T., et al., *Nature* (1987) 328:313-318; Hogan, K., et al. *Genomics* (1994) 24:608-609). This sequence is common to many L-type calcium channel α_1 subunits from various species. A tetravalent multiple antigenic peptide was synthesized by the protein chemistry laboratories of UNC-CH and was used for immunization at a concentration of 500 µg for the initial dose. A boosting dose of 100 µg of peptide was administered twice at 3 week intervals. Antibody response was detected with ELISA using the synthetic peptide as antigen and on Western blots using megakaryocytes solubilized with Laemmli sample buffer and fractionated by SDS-PAGE electrophoresis.

Pig platelets were used at a concentration of 1.5×10^{11}/mL. They were isolated from 1 L of fresh whole blood, pelleted and washed twice in RPMI supplemented with a cocktail of protease inhibitors. Washed platelets were then lysed in a lysis buffer (Boeringer Manheim of Indianapolis, Indiana) which contained 50Mm Tris-HCl, pH 7.5, 150Mm NaCl, 1% Nonidet P40, 0.5% sodium deoxylate and a cocktail of protease inhibitors (supplied by Boeringer Manheim of Indianapolis, Indiana). The platelet lysate was transferred to a pre-chilled Dounce homogenizer and homogenized by approximately 10-15 repeated strokes using a type B pestle. After clearing with centrifugation at 12,000 g the supernate was preabsorbed onto protein A-agarose suspension overnight at 4°C. The beads were discarded, and the supernate was incubated

overnight at 4°C with the purified rabbit anti-peptide IgG at a concentration of 200 µg/mL. Controls were incubated with a non-immune rabbit IgG at the same concentration (Dako Corp. of Carpinteria, California).

The immune-complexes were captured with 100 µg of a protein A-agarose beads overnight, at 4°C. After extensive washings, the immunoprecipitated proteins were separated on SDS-PAGE after eluting the immune complex with Loemmli sample buffer at 100°C for 3-4 min. Western blots were performed after transfer of proteins onto nitrocellulose or PVD membranes. The antipeptide antiserum was used as a primary antibody on western blots at a dilution of 1:500. A goat antirabbit antibody labeled with alkaline phosphatase was used as a secondary antibody. The substrate for the immunoprecipitation reactions were provided in the electrochemiluminescence (ECL) reaction (Amersham Pharmacia Biotech of Piscataway, New Jersey).

To confirm that the antipeptide antibody recognized α_1S and α_1D in human bone marrow megakaryocytes, it was incubated for 60 minutes at r. t. on human bone marrow smears. The antiserum was used in 1:1000 dilution. A fluorescent labeled goat antirabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) was used as a secondary antibody.

To preserve the antigenicity of the determinant recognized by our antipeptide antibody, human platelets were processed using the post-embedding method described by Madden, V. J., *Microscp. Microanal.* (1998) 4(Supp. l2: proceedings):854-855. Fresh human platelets were allowed to settle and adhere on glass coverslips for ten minutes at r. t. Following gentle rinsing with PBS, the platelets were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde in 0.1M sodium cacodylate, pH 7.4, and irradiated using a laboratory microwave oven (Ted Pella, Inc., Redding, California). The cells were postfixed in 1% buffered osmium tetroxide for 10 minutes at r. t. They were dehydrated in acetone and infiltrated with L.R. White resin (London Resin Co., Ltd., Reading, England) using microwave irradiation. After polymerization with ultraviolet light (365 nm wavelength), the monolayers were sectioned *en face* at a thickness of 80 nm and mounted on 300 mesh nickel grids.

For immunocytochemical labeling, all steps were carried out at r. t. The anti-peptide antibody was diluted in 0.1M phosphate-buffered saline with globulin-free 0.1% bovine serum albumin, pH 7.4, and the secondary immunogold labeled antibody (Amersham Life Science Inc. of Arlington Heights, Illinois) was diluted in 0.1M Tris-buffered saline with 0.1% bovine serum albumin, pH 8.2. Grid-mounted sections were etched in 5% aqueous sodium metaperiodate for 30 min, then incubated in 0.2M glycine in PBS/BSA for 5 min. The sections were blocked in 5% normal goat serum in PBS/BSA for 10 min before incubation in the anti-peptide antibody (1:5 dilution) for 2 hours. After rinsing with PBS/BSA, the sections were incubated for 1 hour in goat anti-rabbit IgG 10 nm colloidal gold (1:25 dilution). Negative controls were performed concurrently by deleting primary antiserum and/or incubating in normal rabbit IgG at 1:5 dilution (Dako Corp. of Carpinteria, California).

The immunogold-labeled sections were post-fixed in 1% glutaraldehyde in PBS followed by uranyl acetate and lead citrate treatment. The grids were observed and photographed using a LEO EM-910 transmission electron microscope (LEO Electron Microscopy, Thornwood, New York) at 80 kV. Platelets were also prepared by conventional electron microscopy using 0.1% tannic acid in the fixative so as to obtain the optimal ultrastructural morphology of the surface-connected membranous open canalicular system.

Example 4

Two L-type VDCC α_1 subunit cDNAs are Expressed in Platelets and Megakaryocytes

Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify, clone, and sequence flanking regions of VDCC α_1 subunit cDNA from cultured human megakaryocytes (Meg 01, ATCC) and from fresh porcine platelets. Two different isoforms were found to be expressed in human megakaryocytes. One exhibits sequence identity to the L-type VDCC α_1 subunit sequence from human skeletal muscle α_1S (Hogan, K., et al. *Genomics* (1994) 24:608-609), and is presented herein as SEQ ID NOs:1, 2, 5, and 28. The other exhibits sequence identity to the human neuro-endocrine cells α_1D sequence (Seino, S., et al., *Proc Natl Acad Sci U S A.* (1992) 89:584-588 and Figs. 1A and 1B), and is presented herein as SEQ ID NOs:3, 4, 6, and

29. These partial cDNAs encode regions between III S_4 and the carboxyl-end of α_1S, and between III S_5 and the carboxyl-end of α_1D, respectively (Figs. 1A and 1B). Both cDNAs are missing nucleotides that encode a peptide in the putative extracellular linker between IV S_3 and IV S_4 (Fig. 1B). The intracytoplasmic loop between motifs II-III of the human α_1S was also sequenced (Figs. 1A and 1B).

Similarly, a cDNA was obtained from circulating porcine platelets that corresponds to an encoded peptide in the region between IV S_3 and the amino acid 1531 of α_1S (Figs. 1A and 1B). This platelet cDNA has 91% sequence identity with the rabbit α_1S skeletal muscle subunit (Tanabe T., et al., *Nature* (1987) 328:313-318), is presented herein as SEQ ID NOs:7 and 8 and demonstrates the same missing sequence as the above human sequence in the putative IV S_3 and IV S_4 linker (Figs. 1A and 1B). Another porcine platelet cDNA which encodes the region between II S_3 and II S_6 of the α_1 subunit was sequenced. This partial porcine cDNA has 88% sequence identity to the human neuro-endocrine α_1D cDNA.

The cloned cDNAs indicate that two different α_1 subunits of L-type voltage dependent calcium channels are expressed in the platelet and their megakaryocytic precursors and that they encode polypeptides having sequence identity with the α_1 subunits of VDCCs from human skeletal muscle and neuro-endocrine cells. Importantly, the encoded polypeptides have regions of known active roles in the VDCC functions.

The cloned cDNAs predict the following structural and functional regions:

(a) pore lining segments in III S_5 - S_6 and IV S_5 - S_6 linkers (Figs. 1A and 1B): VDCCs pores are lined by the amphipatic loops in the S_5 - S_6 linkers in each of the four motifs, where a glutamate residue in equivalent positions conveys calcium selectivity to the ion-conducting channel;

(b) voltage sensing segments in transmembrane regions III S_4 and IV S_4 where the positively charged residues in every third or fourth position sense depolarization and induce conformational changes responsible for channel gating;

(c) dihydropyridine binding sites in III S_6 and IV S_6 characteristic of L-type calcium channels (Striessnig J., et al., *Trends Pharmacol Sci.* (1998) 19:108-115); and

(d) an intracytoplasmic loop between II S_6 and III S_1 that is unique to α_1S and believed to be specific for the type of excitation-contraction coupling in skeletal muscle.

The missing sequence in the IV $S_3 - S_4$ linker in every cDNA cloned is envisioned to be a characteristic for the VDCC function in the platelets. A skipped exon has been detected in the same region in other L-type VDCCs. (Peres-Reyes E. and Schneider. T., *Kidney Int.* (1995) 48:1111-1124; Snutch, T. P., et al., *Neuron* (1991) 7:45-57; Diebold, R. J., et al., *Proc Natl Acad Sci U S A.* (1992) 89:1497-1501). Isoform specific riboprobes that correspond to the nucleotides between 3763 and 4796 of the human α_1S and the corresponding sequence from α_1D (Fig. 1C) were synthesized as cmRNA and used to probe separate mRNA blots from human megakaryocytes. A 6.5kb transcript hybridized with the α_1S specific cmRNA, and a 10.8 kb transcript hybridized with the α_1D specific cmRNA on Northern blots (Fig. 1D). The sizes of these transcripts are consistent with those published for α_1S and α_1D from skeletal muscle and neuroendocrine mRNA respectively. Tanabe T., et al., *Nature* (1987) 328:313-318; Seino S., et al., *Proc Natl Acad Sci U S A.* (1992) 89:584-588.

Example 5

α_1S and α_1D VDCC Subunits Are Expressed at the Protein Level in Platelets and Megakaryocytes

An antipeptide antibody was generated in a rabbit against the peptide sequence aa 1486 – 1506 (Tanabe T., et al., *Nature* (1987) 328:313-318; 19, Fig. 1 arrow). This sequence is conserved in all published L-type VDCC α_1 subunits. This antibody recognizes its antigenic determinant in a 170 kDa polypeptide on Western blots from human megakaryocytes resolved by SDS-PAGE (Fig. 2A). This electrophoretic mobility is consistent with that of α_1S on SDS-PAGE previously described from skeletal muscle by Tanabe T., et al., *Nature* (1987) 328:313-318. That the 170 kDa polypeptide is indeed α_1S was confirmed with a monoclonal antibody (Mab 1A) previously published to

recognize its determinant in α_1S from skeletal muscle (Fig. 2A), kindly provided by Dr. S. Froehner, Department of Cell Physiology, UNC-CH and described by Morton, M. E. and Froehner, S. C., *J Biol Chem.* (1987) 262:11904-11907.

Suspecting that α_1D is expressed at a level below the detection threshold on direct Western blots, the subunits were enriched by immunoprecipitation using the anti-peptide antibody described in Example 3 above. Polypeptides with 208 kDa and 170 kDa electrophoretic mobilities were immunoprecipitated from porcine platelets. When the primary antibody in the immunoprecipitation reaction was replaced with non-immune IgG, these polypeptides were not present. See Fig. 2B.

That the anti-peptide antibody recognizes the 208 kDa as α_1D was confirmed by Western blots on pancreatic β cells in culture resolved by SDS-PAGE, as shown in Fig. 2A. Pancreatic β cells in culture were kindly provided by Dr. Michael Freemark, Duke University Department of Pediatrics. The antibody recognized a determinant in a polypeptide with electrophoretic mobility of 208 kDa in the pancreatic β cells. Mab 1A did not recognize a determinant in these cells. The determinant recognized by Mab 1A has not been mapped and thus, might be specific to α_1S.

The expression of the α_1 subunits in *in vivo* megakaryocytes was demonstrated on human bone marrow smears with the anti-peptide antibody as a primary antibody and an FITC labeled anti-rabbit secondary antibody. A discrete speckled pattern was detected on the megakaryocytes and was interpreted to be α_1 subunits. Thus, the anti-peptide antibody recognized its determinant in a speckled pattern. The anti-peptide antibody was also replaced with non-immune rabbit IgG as a negative control. In the analysis, original magnification was 60x.

Thus, the polypeptide that has an electrophoretic mobility of 170 kDa corresponds to the 6.5 kb transcript identified on Northern blots and represents the α_1S subunit from human megakaryocytes and porcine platelets (SEQ ID NOs:1, 2, and 7-8, respectively). In contrast, the 208 kDa polypeptide corresponds to the 10.8 kb transcript on Northern blots and represents the α_1D subunit from human megakaryocytes and porcine platelets.

Example 6

The Platelet VDCC α_1 Subunit Polypeptide α_1 Subunit Is Localized in a Tight Membranous Network of the Open Canalicular System

The site of probable function of the VDCC in the platelet was investigated at the ultrastructural level using the anti-peptide antibody to localize the antigenic determinant in unstimulated human platelets. An anti-rabbit secondary antibody labeled with 10 nm gold particles showed that the epitope recognized by the anti-peptide antibody was present in a tightly branching membranous network inside the platelets. This network forms a tortuous cribriform vesicular membranous system previously described by conventional electron microscopy to be continuous with the surface-connected open canalicular system (White, J. G., *Am J Pathol.* (1970) 58:31-49; White, J. G., *Am J Pathol.* (1972) 66:295-305).

In the examination of localization of VDCC, human platelets were post-embedded and incubated with the anti-peptide antibody as primary antibody. Ten nm gold particle labeled goat anti-rabbit antibody was the secondary antibody. Electron dense gold particles represented antigenic sites recognized by the anti-peptide antibody. This determinant was present in a membranous network continuous with the surface-connected open canalicular system. This network forms tightly branching vacuoles that extend deep inside the platelet. A sample was prepared by conventional EM to maintain the morphological integrity of the surface-connected open canalicular system. No gold particles were present when the anti-peptide antibody was replaced with normal rabbit IgG in a control experiment. In the analysis, original magnification was 16,000x and 500x.

Discussion of Examples

L-type VDCCs are generally co-localized with specialized intracellular organelles of which the function is calcium concentration dependent (Berridge, M. J., *J Physiol (Lond).* (1997) 499.2:291-306; Bokvist K., et al., *EMBO J.* (1995) 14:50-57; López-López, J.R., et al., *Science* (1995) 268:1042-1045; Shirokova, N., et al., *J Physiol (Lond).* 1998: 512.2, 377-384). Activation of the VDCC in the cell membrane generates highly localized signals in the inner microenvironment that trigger responses strictly from the underlying organelles.

Indeed, the L-type VDCC in striated muscle is preferentially localized in the t-tubule membrane, a sequestered surface membrane that invaginates from the cell membrane deep into the muscle cell. There, the VDCC is in close proximity with the calcium storing sarcoplasmic reticulum (SR) of muscle. Activation of the L-type VDCC in the t-tubule triggers the primary signal that causes release of Ca^{2+} from the SR lumen (Hille, B., *Ionic channels of excitable membranes*, Sunderland, MA: Sinauer Associate, Inc. Publishers (1992); López-López, J.R., et al., *Science* (1995) 268:1042-1045; Shirokova, N., et al., *J Physiol (Lond).* (1998) 512.2:377-384). This then generates the global Ca^{2+} signal that results in muscle contraction (Berridge, M. J., *J Physiol (Lond).* (1997) 499.2:291-306).

Similarly, the L-type VDCC is clustered in the cell membrane of insulin secreting β cells in a domain that is adjacent to the highest density of secretory granules inside the cell (Bokvist K., et al., *EMBO J.* (1995) 14:50-57). Activation of the L-type VDCCs in the cell membrane results in a localized intracellular Ca^{2+} burst that initiates exocytosis and release of insulin from these granules.

These morphological relationships in muscle and endocrine cells between the VDCC in surface membrane domains and specialized intracellular organelles appear structurally homologous to those observed in the platelet, as discussed herein above. The surface-connected open canalicular system allows the platelet cell membrane to extend deeply into the platelet. There, some of its components are juxtaposed with the dense tubular system, the calcium storing compartment of the platelet (White, J. G., *Am J Pathol.* (1970) 58:31-49). Others act as a conduit in the platelet secretory pathway (White, J. G., *Am J Pathol.* (1972) 66:295-305). The finding of a preferential localization of the VDCC α_1 subunit in this membranous network implicates the VDCC at these sites in specific functions of the platelet, such as contraction and secretion.

That the L-type voltage dependent calcium channel is physiologically active in platelets is supported by a study on the effects of the dihydropyridine antagonists on platelet aggregation (Palés, J., et al., *Biochem. et Biophys. Acta* (1991) 1064:169-174). When used in a nanomolar range, these L-type calcium

channel blockers inhibit platelet aggregation in a dose-dependent manner. Inhibition of Ca^{2+} channels at these submicromolar concentrations is believed to be selective for the L-type voltage dependent calcium channel (Lee, K.S., et al., *Nature* (1983) 302:790-794). Also, and of interest, is the unexplained finding that platelet aggregation time is prolonged in hypertensive patients receiving an L-type dihydropyridine calcium channel blocker for control of their hypertension (Sinzinger, H., et al., *Eur J Clin Pharmacol.* (1992) 42:43-46; Gebara, O.C., et al., *Clin Cardiol.* (1996) 19:205-211; Tison, P., et al., *Am J Hypertens.* (1994) 7:465-495).

Other studies, namely electrophysiological patch experiments, have not supported the role of VDCC in Ca^{2+} entry in the platelets (Mahaut-Smith, M.P., et al., *J Biol Chem.* (1992) 267:3060-3065) while other electrophysiologic studies have confirmed in phospholipid bilayers the presence of a calcium selective channel in platelet vesicles (Zschauer, A., et al., *Nature* (1998) 334:703-705). The preferential localization of the VDCC, as disclosed herein for the first time, in membranes deep inside the platelets could have masked these channels from patch experiments. Furthermore, the binding and antagonistic effects of dihydropyridines on the L-type VDCC are known to depend on the gated state of the channel. In the resting state this channel has a low affinity for these pharmacological agents (Bean, B.P., *Proc Natl Acad Sci U S A.* (1984) 81:6388-6392). This gated dependent property could complicate the interpretation of results from experiments that use channel blockers on platelet aggregation and could explain some of the discrepancies reported on their effects on platelet function in different assays.

Tissue-specific expression of VDCC α_1 subunits confers a unique function to that tissue or organ. The expression of α_1D in neuroendocrine cells reflects the characteristics of the VDCC in excitation-secretion coupling in these cells. Similarly, the expression of α_1S in skeletal muscle (Birnbaumer L., et al., *Neuron* (1994) 13:505-506; Tanabe T., et al., *Nature* (1987) 328:313-318) or α_1C in cardiac muscle (Birnbaumer L., et al., *Neuron* (1994) 13:505-506; Mikami, A., et al., *Nature* (1989) 340:230-233) reflects the characteristics of the corresponding VDCC in excitation-contraction coupling in skeletal (Shirokova, N., et al., *J Physiol (Lond).* (1998) 512.2:377-384) and cardiac (López-López,

J.R., et al., *Science* (1995) 268:1042-1045) muscles respectively. Extrapolating the expression of α_1D and α_1S subunits to the function of the platelet, the corresponding channels are implicated in excitation-secretion and excitation-contraction couplings in platelets.

REFERENCES

The publications and other materials listed below and/or set forth by author and date in the text above to illuminate the background of the invention, and in particular cases, to provide additional details respecting the practice, are incorporated herein by reference. Materials used herein include but are not limited to the following listed references.

Abbrachio, M. P., et al., *Pharmacol Ther.* (1994) 64:445-475.

Adelman et al., *DNA* 2:183 (1983).

Ahuja et al., *Cancer Res.* (1998) 23:5489-94.

Armstrong, C. M. and Hille, B., *Neuron* (1998) 20:371-380.

Ashcroft, F. M., *Ion channels and disease.* San Diego, California: Academic Press; (2000).

Ausubel et al. (1992) *Current Protocols in Molecular Biology*, (J. Wylie & Sons, N.Y.)

Bean, B.P. *Proc Natl Acad Sci U S A.* (1984) 81:6388-6392.

Bean, B. P., *Trends Phys Sci.* (1993) 13:87-90.

Belinsky et al., *Proc. Natl. Acad. Sci. USA* (1998) 20:11891-6.

Berridge, M. J., *J Physiol (Lond).* (1997) 499.2:291-306.

Bhenke, O. (1992) *J. Submicroscopic Cytology and Pathology* 24:169-178.

Birnbaumer L., et al., *Neuron* (1994) 13:505-506.

Bitgood et al. (1996) *Curr. Biol.* 6, 298-304.

Bodanszky, et al., "*Peptide Synthesis*", John Wiley & Sons, Second Edition, 1976.

Bokvist K., et al., *EMBO J.* (1995) 14:50-57.

Boyd, A. E., *J Cell Biochem.* (1992) 48:234-261.

Crabtree, G. and Henderson, J. (1971) *Cancer Res.* 31:985-991.

Crea et al. (1978) *Proc. Natl. Acad. Sci. U.S.A*, 75:5765.

Diebold, R.J., et al., *Proc Natl Acad Sci U S A.* 1992: 89, 1497-1501.

Eichenlaub et al. (1979) *J. Bacteriol* 138:559–566.

Fields et al., *Int. J. Peptide Protein Res.*, 35:161–214, 1990

Fischer, T., et al. (2000) *Brit. J. Haem.* (in press)

Gebara, O.C., et al., *Clin Cardiol.* (1996) 19:205-211.

Gribskov et al., *Nucl. Acids. Res.* 14:6745 (1986).

Hiki et al. (1999) *J Biol Chem* 274(15):10661-7.

Hille, B., *Ionic channels of excitable membranes*, Sunderland, MA: Sinauer Associate, Inc. Publishers (1992).

Hogan, K., et al. *Genomics* (1994) 24:608-609.

Holmsen, H. (1972) *Ann. N.Y. Acad. Sci* 201:109-115.

Holtzman, E. J., et al., (1998) *Am. J. Physiol.* 275:F550–F564

Hopp, U.S. Patent No. 4,554,101.

Harlow et al. (1988) *Antibodies A Laboratory Manual*, (Cold Spring Harbor Laboratory).

Jin, J. and Kanapuli S., *Proc Natl Acad Sci U S A.* (1998) 95:8070-8074.

Kanapuli, S., *Trends Pharmacol Sci.* (1998) 19:391-394.

Khandelwal, G., et al. (1997) *FASEB J.* 11:1812.

Kyte et al. (1982) *J. Mol. Biol.* 157:105.

Lee, K.S., et al., *Nature* (1983) 302:790-794.

López-López, J.R., et al., *Science* (1995) 268:1042-1045.

Mackenzie, A.B., et al., *J Biol Chem.* 1996: 271, 2879-2881.

Madden, V.J., *Microscp. Microanal.* (1998) 4 (Suppl2: proceedings), 854-855.

Mahaut-Smith, M.P., et al., *J Biol Chem.* (1992) 267:3060-3065.

Makalowski, W., and Boguski, M. S. (1998) *Proc.Natl.Acad.Sci.USA* 95:9407–9412.

Malouf, N. N., et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:5019-5023.

Maniatis et al. (1978) *Cell* 15:687-701.

McOmie, *Protective Groups in Organic Chemistry*, Plenum Press, New York, (1973)

Meienhofer, *Hormonal Proteins and Peptides*, Vol. 2, p. 46, Academic Press, New York, (1983)

Merricks, E., et al. (1998) *Blood* 10(S1-2):71b.

Merrifield (1969) *Adv. Enzymol* 32:221–96.

Messing et al. (1981) *Third Cleveland Symposium on Macromolecules and Recombinant DNA*, Editor A. Walton, (Elsevier, Amsterdam).

Mikami, A., et al., *Nature* (1989) 340:230-233.

Mills, D. C., *Thromb Haemost.* 1996: 76, 835-856.

Morton M.E., Froehner S.C. *J Biol Chem.* 1987: 262, 11904-11907.

Needleman et al. (1970) *J. Mol. Biol.* 48:443.

Novak, E. and Rabinovitch, P. (1994) *Cytometry* 17:135-141.

Ochman et al. (1990) *Amplification of flanking sequences by Inverse PCR, in PCR protocols: a Guide to Methods and Applications* (Innis *et al.*, eds.) pp. 219-227. Academic Press, San Diego, CA.

Palés, J., et al., *Biochem. et Biophys. Acta* (1991) 1064:169-174.

Peres-Reyes, E. and Schneider, T., *Kidney Int.* (1995) 48:1111-1124.

Sage, S. O., *Exp Physiol.* (1997) 82:807-823.

Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

Sanders, W., et al. (1996) *Blood* 88:S107.

Schroder et al., "*The Peptides*", Vol. 1, Academic Press (New York) (1965).

Schwartz et al., eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 357-358 (1979).

Seino, S., et al., *Proc Natl Acad Sci U S A.* (1992) 89:584-588.

Shirokova, N., et al., *J Physiol (Lond).* 1998: 512.2, 377-384.

Sinzinger, H., et al., *Eur J Clin Pharmacol.* (1992) 42:43-46.

Smith et al., *Adv. Appl. Math.* 2:482 (1981).

Snutch, T.P., et al., *Neuron* (1991) 7:45-57.

Steward et al. (1969) *Solid Phase Peptide Synthesis*, W. H. Freeman Co., San Francisco.

Striessnig, J., et al., *Trends Pharmacol Sci.* (1998) 19:108-115.

Strühmer, et al., *Nature* (1989) 339:597-603.

Sun B., et al. *J Biol Chem.* (1998) 273:11544-11547.

Surprenant A., *Trends Neurosci.* (1995) 18:224-229.

Tanabe T., et al., *Nature* (1987) 328:313-318.

Tanabe T., et al., *Nature* (1990) 346:567-569.

Tison, P., et al., *Am J Hypertens.* (1994) 7:465-495.

U.S. Patent No. 5,614,396
U.S. Patent No. 5,589,375
U.S. Patent No. 5,624,816
U.S. Patent No. 5,583,103
U.S. Patent No. 5,580,979
U.S. Patent No. 5,734,033
U.S. Patent No. 5,723,593
U.S. Patent No. 5,739,278
U.S. Patent No. 5,753,687
U.S. Patent No. 5,641,484
U.S. Patent No. 5,693,488
U.S. Patent No. 5,399,346
U.S. Patent No. 5,352,660
U.S. Patent No. 5,326,902
U.S. Patent No. 5,286,634
U.S. Patent No. 5,279,833
U.S. Patent No. 5,234,933
U.S. Patent No. 5,162,215
U.S. Patent No. 5,741,957
U.S. Patent No. 5,120,535
U.S. Patent No. 4,769,331
U.S. Patent No. 4,736,866
U.S. Patent No. 4,686,283
U.S. Patent No. 5,651,964
U.S. Patent No. 5,573,933
U.S. Patent No. 5,550,316
U.S. Patent No. 5,625,125
U.S. Patent No. 4,683,202
U.S. Patent No. 4,244,946
U.S. Patent No. 5,643,567
U.S. Patent No. 4,196,265
U.S. Patent No. 3,095,355
U.S. Patent No. 2,868,691

U.S. Patent No. 5,489,742
U.S. Patent No. 5,436,288
U.S. Patent No. 5,627,158
U.S. Patent No. 5,648,061
U.S. Patent No. 5,646,008
U.S. Patent No. 5,837,479
U.S. Patent No. 5,786,152
U.S. Patent No. 5,776,902
U.S. Patent No. 5,645,999
U.S. Patent No. 5,770,609
U.S. Patent No. 5,780,436
Wetmur & Davidson (1968) *J. Mol. Biol.* 31:349-370.
White, J. G., *Am J Pathol.* (1970) 58:31-49.
White, J. G., *Am J Pathol.* (1972) 66:295-305.
White, T. E., et al. (1990) *Proc. Natl. Acad. Sci.* 87:758-762.
WO 93/25521
WO 96/40276
Yang J., et al., *Nature* (1993) 366:158-161.
Zimmer et al., *Peptides* 1992, pp. 393–394, ESCOM Science Publishers, B. V. 1993.
Zschauer, A., et al., *Nature* (1998) 334:703-705.

It will be understood that various details of the invention can be changed without departing from the scope of the invention. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation--the invention being defined by the claims.